MICROBIAL SULFUR METABOLISM OF HETEROCYCLIC SULFUR COMPOUNDS

TAKASHI OHSHIRO

1996

MICROBIAL SULFUR METABOLISM OF HETEROCYCLIC SULFUR COMPOUNDS

TAKASHI OHSHIRO

1996

CONTENTS

INTRODUCTION

Sulfur compounds occur widely in nature. In the organism, sulfur atoms exist in the amino acids, cysteine and methionine, chondroitin sulfate, thiamin, biotin, glutathione and other cell constituents. In plants and microorganisms, sulfate is usually incorporated into the organism, subsequently reduced enzymatically to sulfite via adenyl sulfate and phosphoadenyl sulfate. A specific bacteria belonging to *Thiobacillus* species can utilize elemental sulfur for cell growth by oxidizing it [1-3]. The number of enzymes involved in the oxidation of elemental sulfur to sulfite have been reported [4-8]. Sulfur: oxygen oxidoreductase of *Thiobacillus ferrooxidans* utilizes ferric ion (Fe^{3+}) as an electron acceptor of elemental sulfur. Under anaerobic conditions in the presence of Fe^{3+}, the enzyme reduces 4 mol Fe^{3+} with 1 mol elemental sulfur to give 4 mol Fe^{2+} and 1 mol sulfite [8].

By sulfite reductase, sulfite is further reduced to hydrogen sulfide which is converted into the thiol of cysteine by cysteine synthase or cystathionine γ-lyase. Methionine is biosynthesized from cysteine through three enzymatic steps. The sulfur of chondroitin sulfate which is found in the connective tissue is derived from phosphoadenyl sulfate. As described above, the biological sulfur metabolisms of elemental sulfur, amino acids and chondroitin sulfate have been studied exclusively. However, many other sulfur compounds having hetero cyclic rings are found in nature; biotin, lipoic acid and thiamin in the organism correspond to them, several antibiotics such as penicillin and cephalosporin have sulfur atoms in their ring structure, and many thiophene compounds exist in fossil fuels. The sulfur metabolism of these compounds have been little elucidated. Among such hetero cyclic sulfur compounds, I focused on dibenzothiophene (DBT) contained in petroleum and biotin which is a vitamin, and studied the enzyme systems involved in the removal of sulfur from DBT, and in the insertion of sulfur into biotin molecule.

Many kinds of sulfur compounds are found in the fossil fuels coal and petroleum, and occupy a few percent due to the place of origin. When these fossil fuels are combusted, sulfur dioxide is released into the atmosphere, causing a world-wide environmental problem, acid rain and air pollution. The conventional technology, hydrodesulfurization is carried out with metal catalysts in the presence of hydrogen gas under high pressure and temperature in the refineries, the efficient metal catalyst has been investigated successively [9-11]. This method has a very broad substrate specificity and actually removes most sulfur compounds, but it is still difficult to eliminate some organosulfur compounds having complicated structures by the present system unless the reaction pressure, catalytic volume and the ratio of hydrogen gas to oil would be increased. The facilities required for such extreme reaction conditions would be very expensive. Current air quality standards have already placed strict limitations on the amounts of sulfurous air emissions allowed, and these regulations may well become even more stringent in the future. Desirable fuel should contain much less sulfur. Therefore, the alternative technology of effective sulfur removal achieved before or after combustion must be established urgently.

Inorganic sulfur exists mainly as iron disulfide, pyrite and marcasite, in coal. The cells of *Thiobacillus ferrooxidans* and *Sulfolobus acidocaldarius* are effective in the removal of pyrite sulfur from coal [12-14]. *Thiobacillus ferrooxidans* has been proposed to selectively adhere to pyrite, resulting in the microbial removal of inorganic sulfur [15, 16]. The removal of organic sulfur from coal has also been attempted for a decade [17-19], but it seems very difficult because the organic sulfur in coal is covalently bound into its large complex structure. Among the organic sulfur compounds present in fossil fuels, DBT and its alkylated derivatives are regarded as representative. There have been several reports on the isolation of DBT-degrading bacteria by use of DBT as a model compound.

Chapter I describes the microbial desulfurization of DBT for the practical use for petroleum. I isolated a bacterium, *Rhodococcus erythropolis* D-1, which grew on DBT as a sole source of sulfur, and estimated its ability to desulfurize DBT by using the resting cells reaction system. Moreover, I also isolated another bacterium, *R. erythropolis* H-2, which utilized DBT in the presence of hydrocarbon, and examined the desulfurization activity of this strain in the presence of *n*-tetradecane.

Chapter II describes the first demonstration of the enzyme system involved in DBT desulfurization of *R. erythropolis* D-1. I verified the cofactor requirement for the enzymatic desulfurization of DBT and purified the enzyme system converting DBT to DBT sulfone.

Another targeting heterocyclic sulfur compound, biotin, called vitamin H, is a water-soluble B vitamins. In bound form, it is distributed widely as a constituent of animal and human cells. Biochemically, D-biotin functions as the carrier of carbon dioxide in enzymatic carboxylation, transcarboxylation and decarboxylation [20] and is involved in important processes such as gluconeogenesis and fatty-acid synthesis [21]. It is used mainly as an animal feed additive and has potential application in human pharmacy. Currently, D-biotin is produced by a complicated method of chemical synthesis [22], and the novel enantioselective synthesis of D-biotin has been reported [23, 24]. However, a significant increase in the microbial process yield may make a bioconversion competitive. In fact, the development of such a process for biotin has generated considerable interest [25-27].

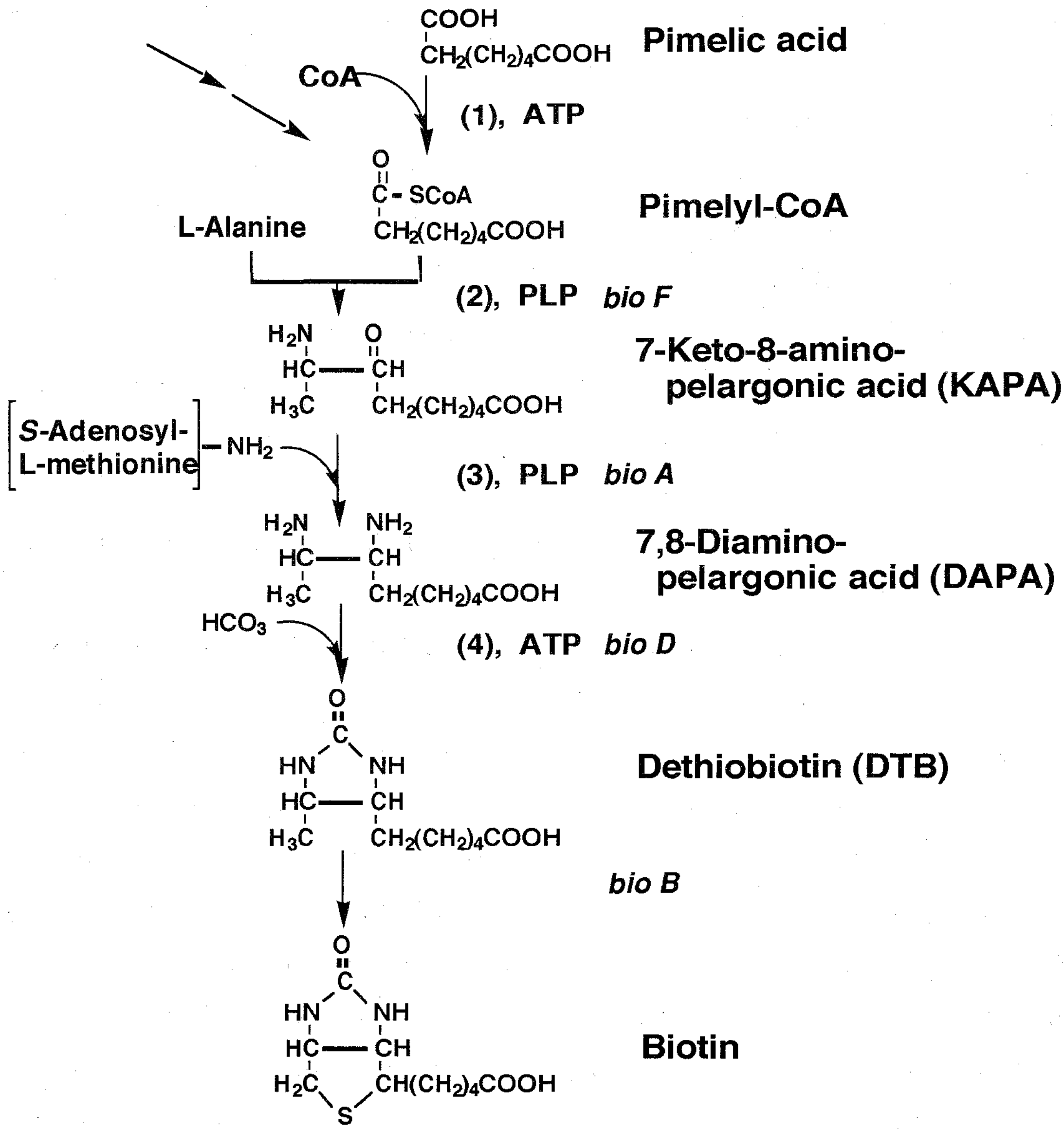


Fig. 1. Biotin Biosynthetic Pathway in Microorganisms.
(1) Pimelyl-CoA synthetase, (2) KAPA synthase
(3) DAPA aminotransferase, (4) DTB synthase
PLP, pyridoxal 5'-phosphate

In microorganisms, biotin is synthesized from pimelyl-CoA as shown in Fig. 1. Pimelyl-CoA has been proposed to be converted from pimelic acid in *Bacillus sphaericus* [28], from alanine and/or acetate via acetyl-CoA in

Escherichia coli [29], and made by a pathway similar to that of fatty acid and polyketide synthesis in *E. coli* [30]. Izumi *et al.* clearly identified all the enzymes involved in biotin biosynthesis from pimelic acid in one bacterium, *B. sphaericus* [31]. Each enzyme reaction is unique because pimelyl-CoA synthetase is a kind of acyl-CoA synthetase which acts on dicarboxylic acid, 7-keto-8-aminopelargonic acid synthase catalyzes the condensation reaction accompanying decarboxylation to form a ketoaminoacid from acyl-CoA and amino acid with pyridoxal phosphate as a coenzyme, 7,8-aminopelargonic acid aminotransferase is a transaminase which requires *S*-adenosylmethionine as an amino donor, dethiobiotin synthase catalyzes carboxylation accompanying the formation of a ureido ring, and biotin synthase incorporates the sulfur atom into biotin. Except for the last enzyme, four enzymes have already been purified and characterized [32-34].

Chapter III deals with the biotin synthase of *B. sphaericus* and *E. coli.* I found out that *S*-adenosylmethionine is an essential cofactor for these enzyme(s), and pyridine nucleotide coenzymes such as NADH or NADPH and FAD enhance the activities. Moreover, I show that the sulfur donor is not *S*-adenosylmethionine but probably cysteine or its derivatives.

CHAPTER I

Microbial Desulfurization of Dibenzothiophene

Section 1. Selective Desulfurization of Dibenzothiophene by *Rhodococcus erythropolis* D-1

Microbial degradation of organic sulfur compounds has attracted attention for its potential application to the desulfurization of coal and petroleum. Among the organic sulfur compounds present in the fossil fuels, dibenzothiophene (DBT) and its derivatives are regarded as representatives. There have been several reports on the isolation of DBT-degrading bacteria by use of DBT as a model compound. A *Brevibacterium* sp. strain mineralized DBT as a sole source of carbon, sulfur, and energy via benzoate to carbon dioxide, sulfite, and water [36]. The partial microbial degradation of DBT was demonstrated by other researchers, and metabolites derived from the oxidation of DBT were detected, depending on the strain and culture conditions. Under aerobic conditions, 3-hydroxy-2-formyl benzothiophene was formed by a *Pseudomonas* sp. [37] and 1,2-dihydroxy-1,2-dihydrodibenzothiophene and dibenzothiophene 5-oxide were generated by *Beijerinckia* sp. [38], and under anaerobic conditions, biphenyl was formed by *Desulfovibrio desulfuricans* M6 [39]. The partial DBT degradation product, 3-hydroxy-2-formyl benzothiophene was degraded completely by a mixed culture [40]. Two bacterial strains, *Corynebacterium* sp. SY1 [41] and *Rhodococcus rhodochrous* IGTS8 [42], were shown to remove only sulfur from DBT, converting DBT to 2-hydroxybiphenyl (2-HBP). These strains appear promising for their ability to selectively remove organic sulfur without the loss of carbon atoms, which bring about energy. Figure 1 shows the proposed pathways of DBT microbial degradation reported previously.

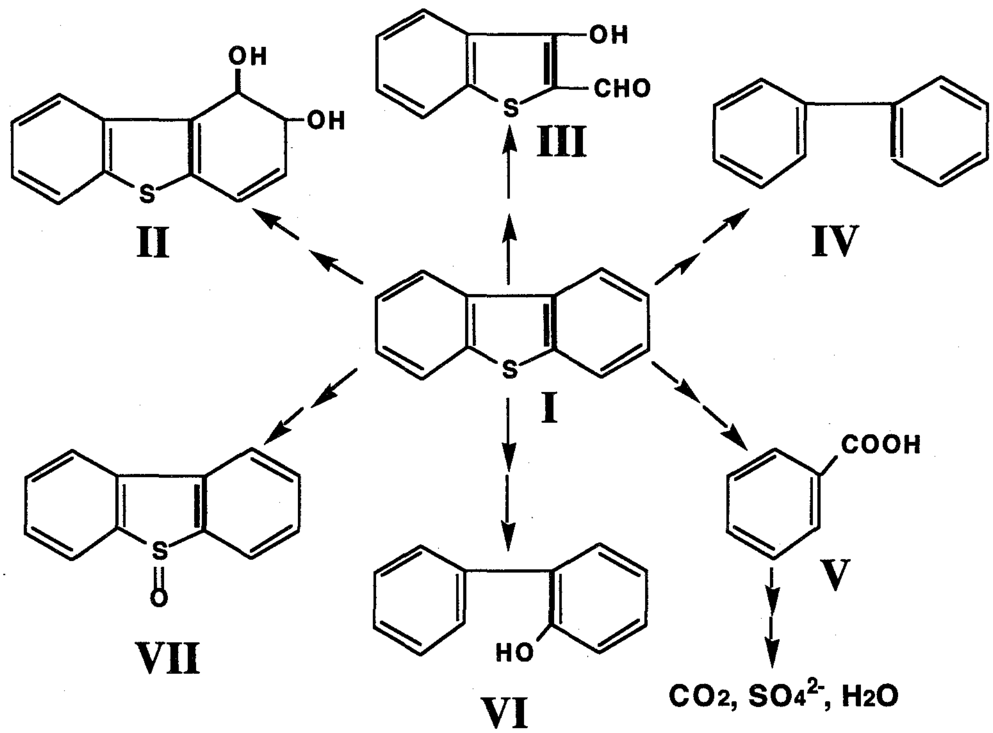


Fig. 1. Proposed Pathways of DBT Degradation.
I: dibenzothiophene (DBT), II: 1,2-dihydroxy-1,2-dihydrodibenzothiophene,
III: 3-hydroxy-2-formylbenzothiophene, IV: biphenyl, V: benzoic acid,
VI: 2-hydroxybiphenyl,
VII: dibenzothiophene 5-oxide

I have isolated a DBT-degrading bacterium, tentatively identified as *Rhodococcus erythropolis* D-1, which utilizes DBT as a sole source of sulfur. This section describes the desulfurization of DBT by growing and resting cells of strain D-1 and compares the DBT-desulfurizing ability and the DBT metabolic pathway of *R. erythropolis* D-1 with those of *R. rhodochrous* and *Corynebacterium* sp.

MATERIALS AND METHODS

Microorganisms. Several strains which utilized DBT as a sole source of sulfur were isolated from 150 soil samples from various areas in Japan by the selective enrichment method with medium A supplemented with DBT at 5.4 mM. Single-colony isolation was repeated on the same medium containing 1.5% agar and finally carried out on a nutrient agar medium. Among the DBT-utilizing strains, strain D-1 was used throughout this work. The strain was maintained on slants of medium A supplemented with DBT at 5.4 mM containing 1.5% agar.

Media and growth conditions. Medium A is a sulfur-free and synthetic medium containing 5 g of glucose, 0.5 g of KH_2PO_4, 4 g of K_2HPO_4, 1 g of NH_4Cl, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.02 g of $CaCl_2$, 0.01 g of NaCl, 10 ml of metal solution, and 1 ml of vitamin mixture in 1000 ml of distilled water, pH 7.5. The metal solution contained 0.5 g of $FeCl_2 \cdot 4H_2O$, 0.5 g of $ZnCl_2$, 0.5 g of $MnCl_2 \cdot 4H_2O$, 0.1 g of $Na_2MoO_4 \cdot 2H_2O$, 0.05 g of $CuCl_2$, 0.05 g of $Na_2WO_4 \cdot 2H_2O$ and 120 mmol of HCl in 1000 ml of distilled water. The vitamin mixture was composed of 400 mg of calcium pantothenate, 200 mg of inositol, 400 mg of niacin, 400 mg of pyridoxine hydrochloride, 200 mg of *p*-aminobenzoic acid, and 0.5 mg of cyanocobalamin in 1000 ml of distilled water. Unless otherwise indicated DBT was dissolved in ethanol (27 mM) and added to sterilized medium A. The nutrient agar medium was composed of 10 g of peptone, 7 g of meat extract, 5 g of yeast extract, 3 g of NaCl, and 15 g of agar in 1000 ml of distilled water. Cultivation was carried out at 30°C in test tubes containing 5 ml of medium with reciprocal shaking (300 strokes/min) or in 2-liter flasks containing 500 ml of medium with reciprocal shaking (100 strokes/min).

Resting cell reactions. Strain D-1 was grown in medium A containing DBT at 0.136 mM in 2-liter flasks for 2 days. Cells were harvested at 4°C by centrifugation at 10,000 x *g* for 15 min, washed once with 0.85% NaCl, and stored at -20°C until use. The cells were suspended in 0.1 M potassium phosphate buffer, pH 7.0. DBT-ethanol solution was added to 1 ml of the cell suspension in a test tube (18 by 180 mm) and the resting cells reaction was allowed to proceed at 30°C with reciprocal shaking at 300 strokes/min.

Analytical methods. Growth was measured turbidimetrically at 660 nm. Determination of DBT and its metabolites was carried out by gas chromatography. The culture broth or the reaction mixture with resting cells in a test tube was acidified to pH 2.0 with 1N HCl and extracted with an 0.8 volume of ethylacetate. A portion of the ethylacetate layer was removed and centrifuged, and 5 μl of the supernatant was injected into a gas chromatograph (GC-14A; Shimadzu, Kyoto, Japan) with a flame ionization detector. The gas chromatograph was equipped with a glass column (3.2 mm by 1 m) packed with Silicone OV-17, 2 % Chromosorb WAWDMCS 80/100 (GL Sciences, Tokyo, Japan). The flow rate of the nitrogen carrier gas was 15 ml/min. The column temperature was programmed from 120 to 250°C at 5°C/min. The injector and detector temperatures were maintained at 250 and 335°C, respectively. DBT and its metabolites were also determined by high-performance liquid chromatography (HPLC) with an 880-PU pump and 875-UV variant wavelength UV monitor (both from JASCO, Tokyo, Japan) and a Lichrospher 100 RP-18 column (5 μm, 4 by 250 mm; Merck, Darmstadt, Germany). Detection was based upon absorption at 280 nm. The mobile phase was 20 mM potassium dihydrogenphosphate, the pH of which was adjusted to 2.5 by phosphoric acid/methanol, 1 : 4 (by vol). The flow rate was 1 ml/min. Sulfate was determined with $BaCl_2$ by the method of Dodgson [43].

Chemicals. DBT was obtained from Wako Chemicals, Osaka, Japan. 2-HBP was purchased from Nacalai Tesque, Kyoto, Japan. DBT sulfone was obtained from Aldrich, Milwaukee, USA. All other chemicals were of analytical grade and commercially available and were used without further purification.

RESULTS

Taxonomic characteristics of strain D-1

Among the isolated strains, one strain, designated D-1, showed the most potent DBT-utilizing ability. Taxonomic characteristics of the strain were investigated at the National Collection of Industrial and Marine Bacteria Ltd. (Aberdeen, United Kingdom). Strain D-1 was a Gram-positive, immotile bacterium which formed coccoid and short rod cells. The strain was catalase-positive and oxidase-negative, and did not produce acid from glucose fermentatively. The strain grew at 37°C but not at 41°C. Colonies of the strain on nutrient agar were round, regular, entire, smooth, slightly mucoid, pale orange-pink, opaque, and low convex. The diamino acid of the cell wall of the strain was meso-diaminopimelic acid. Cells of the strain contained mycolic acids and 10-methyl octadecanoic acid (tuberculostearic acid). The strain utilized inositol, mannitol, sorbitol, sodium adipate, sodium citrate, glycerol, trehalose, *p*-hydroxybenzoic acid, D-mannose, acetamide, sodium lactate and sodium glutamate, but not maltose, rhamnose, *m*-hydroxybenzoic acid, sodium benzoate, L-tyrosine and D-galactose as a sole source of carbon. Therefore, although its maltose and mannose utilization was atypical, I tentatively identified the strain as *Rhodococcus erythropolis*.

DBT utilization by strain D-1

Figure 2 shows the time course of DBT-utilization by the strain in medium A supplemented with DBT at 0.125 mM as a sole source of sulfur. The strain showed maximum growth (about 7units of optical density at 660 nm) at 42 h of

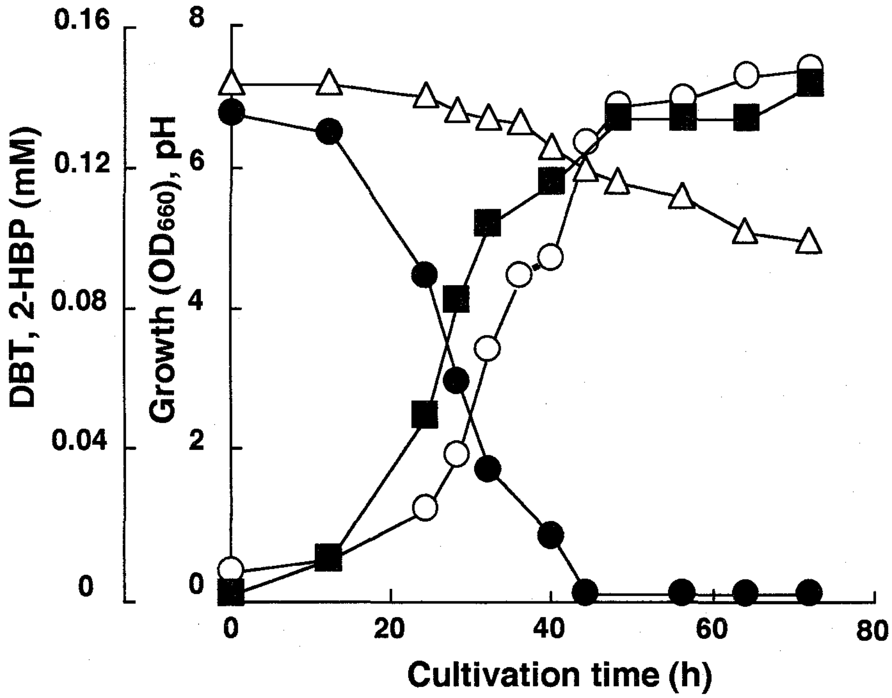


Fig. 2. DBT Utilization by *R. erythropolis* D-1.
The strain was cultivated in medium A supplemented with 0.125 mM DBT as a sole source of sulfur. Symbols: ○, Growth; △, pH; ●, DBT; ■, 2-HBP.

cultivation, concurrent with the depletion of DBT. A metabolite detected in ethylacetate extracts of the cultures was identified as 2-HBP by co-chromatography with authentic 2-HBP by gas chromatography, and by HPLC. 2-HBP accumulation was almost equimolar to DBT depletion in the culture, and did not decrease during the stationary-phase cultivation up to 75 h (Fig. 2). This fact indicated that 2-HBP is a dead-end metabolite of DBT degradation by this strain. In addition to 2-HBP, a metabolite detected in trace amounts by gas chromatography and HPLC was identified as DBT sulfone.

There was a transient appearance of DBT sulfone in cultures grown on DBT between the early exponential and the late log phases of growth. The pH decreased during the cultivation.

Table I. Sulfur Compounds Specificity of *R. erythropolis* D-1
Each sulfur compound was added to medium A at an initial concentration of 25 mg/liter. Volatile compounds, thiophene, 2-methylthiophene, 3-methylthiophene, 2-ethylthiophene, and benzothiophene, were fed to the cultures at a concentration of 25 mg/liter again after 3 days of cultivation.

Sulfur compounds	3 days		7 days	
	Growth (OD_{660})	pH	Growth (OD_{660})	pH
None	0.1	7.2	0.1	7.1
DBT	6.4	4.9	6.6	4.5
DBT sulfone	3.0	6.5	5.0	6.0
Thiophene	0.3	7.1	0.6	6.9
2-Methylthiophene	0.1	7.2	0.2	7.1
3-Methylthiophene	0.1	7.2	0.1	7.2
2-Ethylthiophene	0.2	7.2	0.3	7.1
Benzothiophene	0.1	7.1	0.2	7.1
Thiophene 2-carboxylic acid	0.1	7.2	0.1	7.2
Thiophene 2-acetic acid	0.1	7.2	0.1	7.2
Thianthrene	0.2	6.9	0.5	6.7
Thioxanthen-9-one	1.1	6.6	4.1	6.4
Benzenesulfonic acid	0.1	7.2	0.1	7.2
Benzenesulfinic acid	0.1	7.2	0.1	7.2
p-Toluenesulfonic acid	0.1	7.2	0.1	7.2
Dimethyl sulfate	0.1	7.2	0.2	7.2
Dimethyl sulfone	5.8	5.2	6.8	4.1
Dimethyl sulfoxide	0.1	7.2	0.1	7.2
Dimethyl sulfide	0.2	7.2	0.1	7.2
Methanesulfonic acid	5.3	4.2	6.1	4.1
2-Mercaptoethanol	4.1	5.4	4.8	4.7
Ammonium sulfate	4.3	6.1	5.1	5.8
Sulfur	4.8	6.1	5.8	5.8

Growth specificity of alternate sulfur sources

The strain was cultivated in medium A with 25 mg of various sulfur compounds per liter as a sole source of sulfur for 7 days (Table I). The strain grew well on DBT, DBT sulfone, thioxanthen-9-one, dimethyl sulfone, methanesulfonic acid, 2-mercaptoethanol, elemental sulfur, and ammonium sulfate, all yielding a decrease in the pH of the medium. However, the growth of the strain on the thiophene derivatives tested was negligible.

DBT degradation by resting cells

The desulfurization of DBT was investigated with various amounts of resting cells (Fig. 3). The conversion of DBT to 2-HBP appeared the most

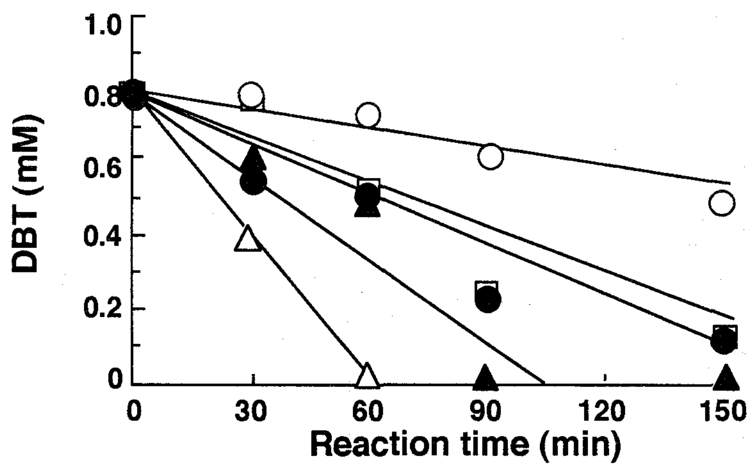


Fig. 3. Effect of Cell Density on DBT Degradation by Resting Cells of *R. erythropolis* D-1.

The reaction mixture contained DBT at an initial concentration of 0.8 mM. Cell density: ○, 4.2; ●, 11; △, 21; ▲, 42; □, 84 mg (as dry weight)/ml.

efficient in reaction mixtures containing 21 mg of cells. It was almost stoichiometric in the resting cells reaction. However, in the reactions with a much higher cell density, DBT degradation was observed to be significantly retarded. This fact may indicate that oxygen is a limiting factor for DBT degradation by the cells. The availability of a water-insoluble substrate (DBT) to the resting cells population may cause the scatter of data points in Fig. 3. Sulfate was detected in the reaction mixtures with resting cells, but the amount of sulfate detected was small and not stoichiometric to the amount of DBT converted to 2-HBP. DBT degradation in reaction mixtures containing various amounts of DBT was examined. DBT up to 2.2 mM (approximately 400 mg/liter) was completely degraded within 150 min as shown in Fig. 4. Almost the same degradation rate was observed in the reactions up to about 3 mM DBT. It indicated no substrate inhibition by DBT.

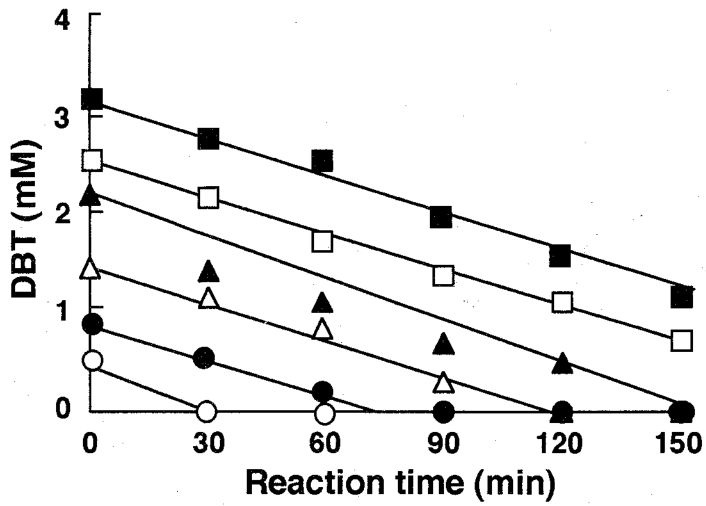


Fig. 4. Effect of DBT Concentration on DBT Degradation by Resting Cells of *R. erythropolis* D-1.

The reaction mixture contained 21 mg of cells (as dry weight)/ml.

DISCUSSION

A new DBT-degrading bacterium, tentatively identified as *R. erythropolis* D-1, utilizes DBT as a sole source of sulfur. The strain showed efficient DBT-desulfurizing ability. DBT at an initial concentration of 0.125 mM was completely degraded within 2 days by growing cells; whereas, 2.2 mM DBT was converted to 2-HBP within 150 min by resting cells. Such an efficient conversion of DBT has not been reported for other DBT-oxidizing microorganisms. For example, it took 48 h that 5.4 mM DBT was reduced to one-tenth by the resting cells of *R. rhodochrous* IGTS8 [42], but detailed reaction conditions were not identical.

The metabolism of DBT by aerobic microorganisms can be generally classified into three types. In type 1 metabolism, the carbon skeleton of DBT is partially oxidized, with the C-S bond remaining intact [44]. In type 2 metabolism, DBT served as the sole source of carbon, sulfur, and energy, with mineralization proceeding through benzoate [36]. In type 3 metabolism, DBT served as the sole source of sulfur. DBT is desulfurized by the selective cleavage of the C-S bond, resulting in the accumulation of 2-HBP [41, 42, 45]. This study shows that DBT degradation by strain D-1 belongs to type 3 metabolism. DBT was stoichiometrically converted to 2-HBP by this strain, indicating that 2-HBP represents a dead end metabolite of DBT metabolism. Sulfate production during the degradation of DBT by resting cells was stoichiometric to the amount of DBT converted to 2-HBP, probably because sulfate was incorporated by the cells. This strain did not grow on DBT as a sole source of carbon. In addition to 2-HBP, a trace amount of DBT sulfone was detected in cultures grown on DBT. This strain utilized DBT sulfone as a sole source of sulfur. In the resting cells reaction 1 mM DBT sulfone added was converted to 0.3 mM 2-HBP after 120 min, and no DBT was detected as a by-product. Therefore, DBT degradation by this strain was assumed to proceed from DBT to 2-HBP via DBT sulfone. The metabolic pathway is similar to that proposed for DBT degradation by *Corynebacterium* sp. SY-1 [41] and *R. rhodochrous* IGTS8 [42, 45]. With this type of DBT degradation, there is no loss of the carbon atoms of DBT, and only DBT sulfur atom was eliminated. This type of DBT degradation may be of potential advantage for the microbial desulfurization of fossil fuels, and it can be extended to other DBT derivatives which are actual forms of DBT present in the fuels.

SUMMARY

A DBT-degrading bacterium, *R. erythropolis* D-1, which utilized DBT as a sole source of sulfur was isolated from soil. DBT was metabolized to 2-HBP by the strain and 2-HBP was almost stoichiometrically accumulated as the dead-end metabolite of DBT degradation. DBT degradation by this strain was shown to proceed as DBT→DBT sulfone→2-HBP. DBT at an initial concentration of 0.125 mM was completely degraded within 2 days cultivation. DBT up to 2.2 mM was rapidly degraded by resting cells within only 150 min. It was thought this strain had a higher DBT-desulfurizing ability than other microorganisms reported previously.

Section 2. Microbial Desulfurization of Dibenzothiophene in the Presence of Hydrocarbon

In Section 1, I isolated a DBT-degrading bacterium, *Rhodococcus erythropolis* D-1, which has the sulfur-specific pathway. Many research groups have since studied the desulfurization of DBT by the sulfur-specific pathway as shown in Fig. 1 [42, 46, 47]. The genes involved in DBT degradation have been identified [48]. However, there has so far been little report on the DBT desulfurization in the presence of hydrocarbon. Since petroleum should ideally be desulfurized, I isolated a strain with the capacity to desulfurize DBT in the presence of hydrocarbon. Here I describe the desulfurization of DBT by growing and resting cells of *Rhodococcus erythropolis* H-2 in the presence of *n*-tetradecane (TD) and other hydrocarbons.

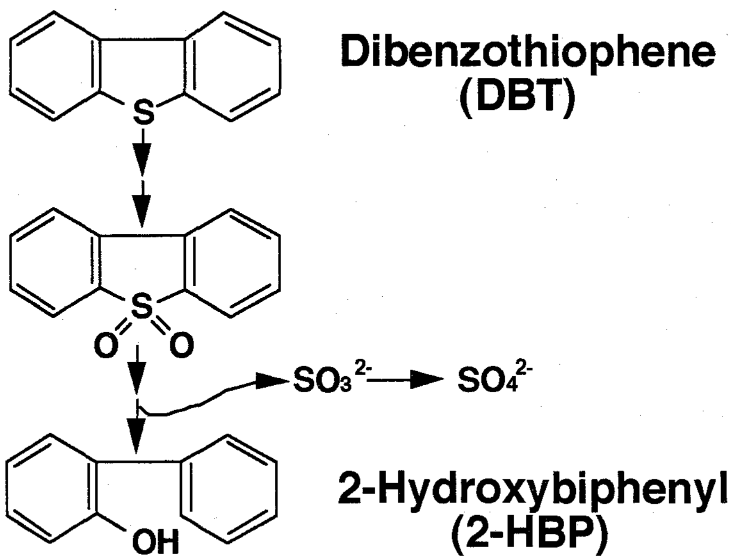


Fig. 1. Proposed Sulfur-specific Pathway of DBT Desulfurization.

MATERIALS AND METHODS

Medium and cultivation. Medium A-1 was the same as medium A described in Section 1 of this chapter except that glucose was omitted. Cells were cultivated at 30°C in test tubes containing 5 ml of medium or in 2-liter flasks containing 500 ml of medium with reciprocal shaking (300 rpm for test tubes and 100 rpm for flasks).

Microorganisms. To isolate bacteria which could desulfurize DBT in petroleum, several soil samples from various areas in Japan were transferred to test tubes containing medium A-1 supplemented with 5.4 mM DBT as a sole source of sulfur and 0.5% TD. Single colony isolation was repeated on the same medium containing 1.5 % agar. Among the DBT-utilizing strains in the presence of TD, I selected strain H-2.

Resting cells reaction. Strain H-2 was cultivated in medium A-1 with 0.5 % glucose and 0.27 mM DBT in 2-liter flasks for 2 days. Cells were harvested at 4°C by centrifugation at 10,000 x *g* for 15 min, washed once with 0.85 % NaCl and resuspended in the same solution. The suspension was lyophilized and kept at -20°C until use. The reaction mixture contained, in 1 ml, TD, DBT which was dissolved in TD, 0.1 M potassium phosphate buffer (pH 7.0) and lyophilized cells. The reaction proceeded in test tubes at 30°C with reciprocal shaking (300 rpm).

Analyses. DBT and 2-HBP were determined by gas chromatography or high performance liquid chromatography as described in Section 1 of this chapter. TD was measured by gas chromatography under the same conditions. When the strain was cultivated in the medium with hydrocarbon, the cells floated on the surface of the medium. Therefore, growth could not be measured turbidimetrically. The culture broth was centrifuged at 15,000 x *g* for 45 min and the cell pellet was resuspended in 0.85% NaCl containing 5% polyoxyethylene lauryl alcohol ether (Brij 35). Cell growth was determined by measuring the optical density of this suspension. The OD_{660} was proven to be proportional to the number of viable cells.

Chemicals. DBT was purchased from Wako Chemicals, Osaka, Japan, and 2-HBP was obtained from Nacalai Tesque, Kyoto, Japan. All other reagents were of analytical grade, commercially available, and used without further purification.

RESULTS

Characterization of a DBT-utilizing bacterium in the presence of hydrocarbon

Among the isolates, a strain designated H-2 utilized DBT most rapidly in the presence of TD. The taxonomic properties were examined at the National Collection of Industrial and Marine Bacteria Ltd. (Aberdeen, Scotland, United Kingdom). As a result, the strain was identified as *Rhodococcus erythropolis*. There are some differences between previously described strain D-1 and the present strain H-2 in grown on carbon source such maltose, L-tyrosine and D-mannose: in strain H-2, these were possibly positive. Since this strain assimilated TD as a carbon source in addition to DBT as a sulfur source, several hydrocarbons were investigated to determine whether or not they could support the growth of *R. erythropolis* H-2. As shown in Table I, this strain grew on *n*-alkanes with carbon chains longer than C_8 with and without glucose, whereas it did not grow on *n*-hexane, styrene, *p*-xylene, cyclooctane and toluene even in the presence of glucose.

Table I. Growth in the presence of hydrocarbons
The strain was cultivated in medium A-1 with 0.5% hydrocarbons with or without 0.5% glucose in test tubes for 4 days.

Hydrocarbon	(log P value)	+Glucose		−Glucose	
		Growth (OD_{660})	pH	Growth (OD_{660})	pH
None		4.8	4.6	n.t [a)]	n.t [a)]
n-Hexadecane	(7.0<)	4.9	4.2	5.2	5.1
n-Tetradecane	(7.0<)	5.6	3.8	4.9	3.8
n-Dodecane	(7.0)	2.9	4.2	3.0	4.8
n-Decane	(6.0)	1.7	5.7	2.5	6.4
n-Nonane	(5.5)	0.8	5.7	0.8	5.8
n-Octane	(4.9)	0.3	6.5	0.3	6.6
Cyclooctane	(4.5)	0	6.9	0	6.9
n-Hexane	(3.9)	0	7.0	0	6.9
p-Xylene	(3.1)	0	7.0	0	7.0
Styrene	(2.9)	0	6.9	0	6.9
Toluene	(2.8)	0	6.9	0	7.0

[a)] not tested

Growth of *R. erythropolis* H-2 in the medium containing DBT and TD

The strain was cultivated in medium A-1 with TD as a sole source of carbon and DBT as a sole source of sulfur. Figure 2 shows the typical growth profile and the time course of DBT and TD utilization. The strain showed maximal growth (OD_{660}=ca. 3.0) after 2 days of cultivation. DBT completely disappeared before this point. The metabolite 2-HBP was formed from DBT and it was almost equimolar to the amount of DBT degraded. The level of TD decreased slightly, and the pH decreased concomitantly with the increase of cell growth.

DBT degradation by resting cells reactions

To prepare resting cells for DBT degradation, *R. erythropolis* H-2 was cultivated in medium containing either 0.5% glucose or 0.5% TD as a carbon source. Cells were lyophilized after harvesting and used for each reaction by resting cells. When the resting cells reactions proceeded with 50% TD for 4 h,

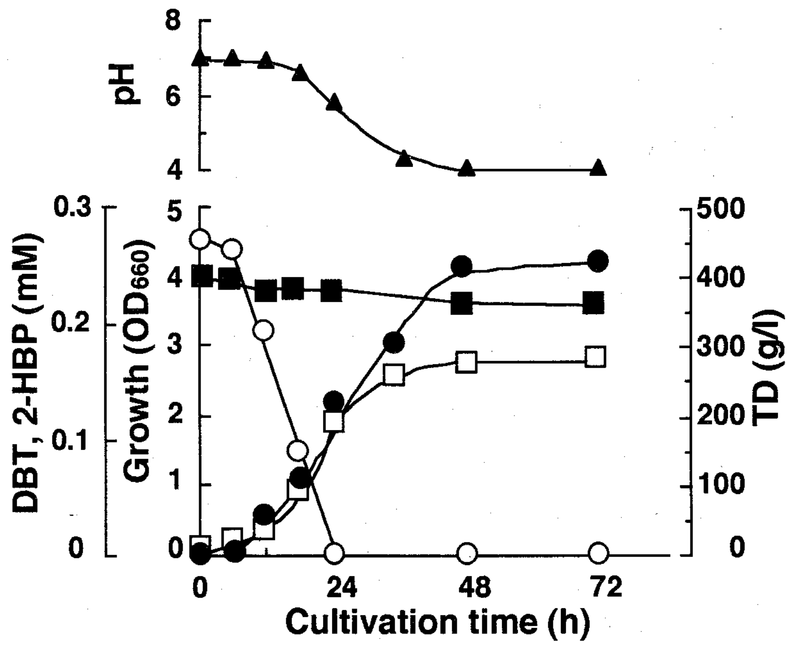


Fig. 2. Cell Growth of *R. erythropolis* H-2 and DBT Utilization during Cultivation.

The strain was cultivated in medium A-1 supplemented with 40% TD and 0.27 mM DBT in test tubes: □, growth (OD_{660}); ○, DBT; ●, 2-HBP; ■ ,TD; ▲, pH.

the DBT degradation rates by cells pregrown in glucose and TD were 60 and 33%, respectively. Therefore, the following studies of resting cells reactions were performed using cells grown in glucose. DBT degradation was investigated using various amounts of lyophilized cells. As shown in Fig. 3, the reaction proceeded most efficiently when the cells were added to the reaction mixture at a concentration of 80 mg/ml. However, DBT degradation was suppressed in the reaction mixture at elevated concentrations of the lyophilized cells. The limitation of oxygen may lower DBT degradation as found in *R. erythropolis* D-1. DBT degradation in reaction mixtures containing various amounts of TD was examined. The reaction proceeded more efficiently with, than without TD (Fig. 4).

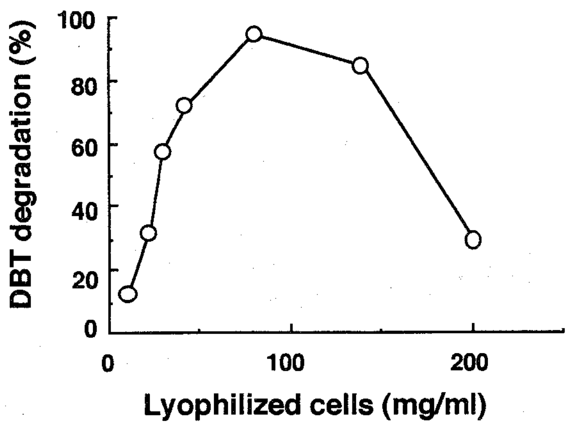


Fig. 3. Effects of Cell Concentrations.
The reaction was done with various amounts of lyophilized cells and 1 mM DBT in 50% TD for 4 h.

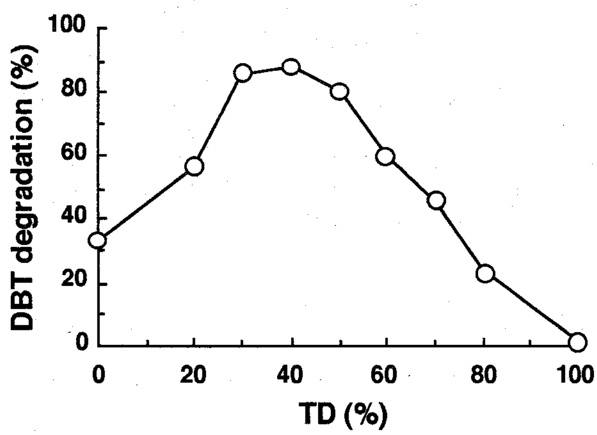


Fig. 4. Effects of TD Concentrations.
The reaction was done with 80 mg/ml of lyophilized cells and 1 mM DBT in various amounts of TD for 1 h.

Even with as much as 70% TD, the degradation was enhanced compared with the situation without TD. The optimal concentration of TD was about

40%. TD at a concentration higher than 80% suppressed the degradation. In a reaction mixture supplemented with 40% TD and 80 mg/ml of the lyophilized cells, DBT up to 3 mM was completely degraded within 4 h. Figure 5 shows the time course of DBT degradation and 2-HBP accumulation. The amount of 2-HBP formed was almost stoichiometric to that of DBT degraded. It seemed that the level of TD was slightly decreased.

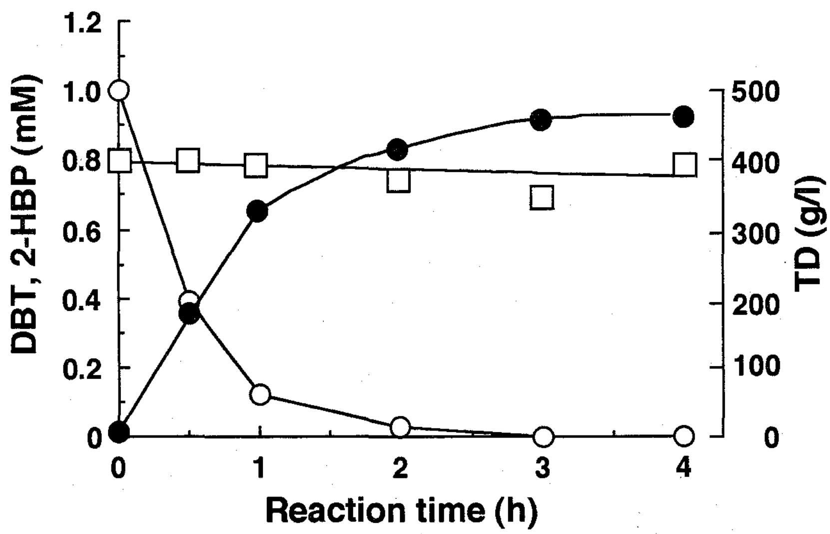


Fig. 5. Time Course of DBT Degradation and 2-HBP Accumulation. The reaction was done with 80 mg/ml of lyophilized cells and 1 mM DBT in 50% TD : ○, DBT, ●, 2-HBP, □, TD.

DISCUSSION

A new strain, identified as *R. erythropolis* H-2, utilized DBT as a sole source of sulfur and converted it to 2-HBP stoichiometrically even in the presence of hydrocarbon. This strain grew well in *n*-alkanes with relatively long carbon chains but not in hydrocarbons with higher toxicity to the organism such as toluene (Table I). The solvent parameter, log P [49, 50], which is defined as the logarithm of a solvent partition coefficient in a standard control octanol : water mixture, was used as the index of a solvent's degree of toxicity. The limiting log P value for the growth of the isolated strain was about 4.9 (log P value of *n*-octanol). From other experimental data [51], *R. erythropolis* H-2 revealed high tolerance against solvents compared with other Gram-positive bacteria, but it had less tolerance than Gram-negative bacteria such as *Pseudomonas* strains. Also in the resting cells reactions, DBT degradation proceeded in the presence of hydrocarbon and was enhanced by adding TD. TD may facilitate contact between DBT and cells since DBT is water immiscible.

Since there is considerable interest in the microbial desulfurization of fossil fuel oil, several reports have been published that describe microorganisms which convert DBT to 2-HBP [41, 47, 52, 53]. However, few studies have centered on the microbial desulfurization in a water-oil mixed system. It has been demonstrated that *Desulfovibrio desulfuricans* M6 reduced organic sulfur in petroleum to sulfide under anaerobic condition using electrochemically supplied electrons [54]. DBT is reportedly better degraded aerobically by *Pseudomonas* sp. when the medium contains *n*-dodecane [55]. However, no detailed conditions were examined: optimal concentration of hydrocarbon, time-course of DBT degradation and metabolites from DBT. Thus, it should be noted that the bacterium, *R. erythropolis* H-2, can efficiently function in the presence of hydrocarbon and desulfurize DBT to form 2-HBP. Therefore, *R. erythropolis* H-2 should be useful for the practical microbial desulfurization of petroleum.

SUMMARY

The bacterium, *R. erythropolis* H-2, which can utilize DBT as a sole source of sulfur in the presence of hydrocarbon, was isolated from soil

samples. When this strain was cultivated in a medium containing 0.27 mM DBT and 40% TD, DBT was metabolized stoichiometrically to 2-HBP within 1 day. This strain grew in the presence of *n*-octane and longer carbon-chain hydrocarbons, but not in that of *n*-hexane, styrene, *p*-xylene, cyclooctane and toluene. DBT degradation proceeded in the resting cells system with lyophilized cells of this strain. The addition of TD enhanced the reaction rate; the optimal concentration of TD was 40%. DBT degradation occurred in the reaction mixture even at a rate as high as 70% TD, whereas TD at concentrations above 80% suppressed the degradation.

Section 3. Microbial Desulfurization of Substituted Dibenzothiophene by *Rhodococcus erythropolis* H-2

DBT is widely recognized as a model organic sulfur compound. It has been demonstrated that some bacterial strains, *Rhodococcus erythropolis* D-1 described in Section 1 of this chapter, *R. rhodochrous* IGTS8 [42, 46, 53], *Corynebacterium* SY-1 [41] and *Agrobacterium* sp. [54] can grow on DBT as a sole source of sulfur. In practice, however, many kinds of DBT derivatives such as alkyl-substituted DBT exist in petroleum, it was thought that more complicated compounds were more recalcitrant to the desulfurization process. Although many researchers have studied the desulfurization of DBT described above, no reports have appeared concerning the desulfurization of alkyl-substituted DBT. I isolated *R. erythropolis* H-2 which could desulfurize DBT in the presence of hydrocarbon as described in the previous section. In this section, I describe the desulfurization of substituted DBT, as shown in Fig. 1, by *R. erythropolis* H-2.

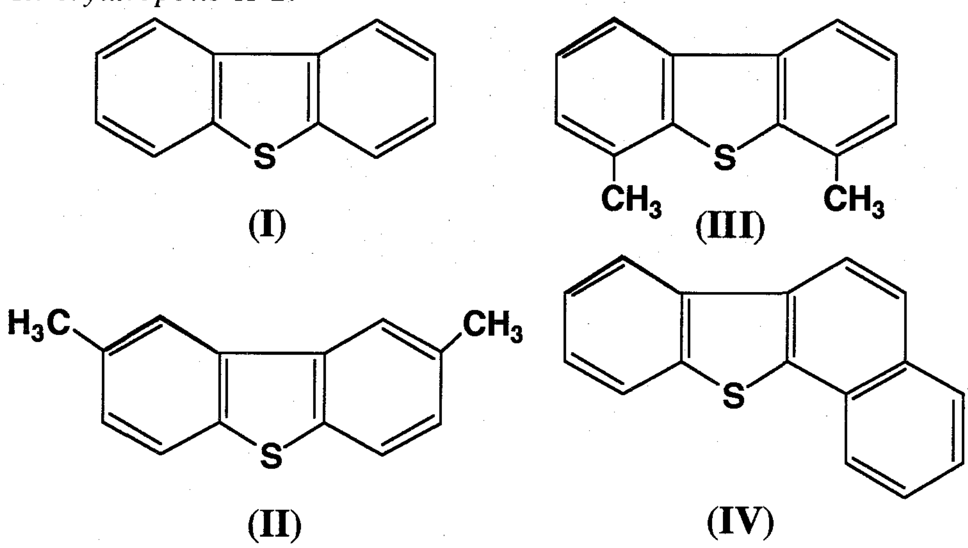


Fig. 1. Structure of DBT and its Derivatives.
(I); DBT, (II); 2,8-dimethyl DBT, (III); 4,6-dimethyl DBT, (IV); 3,4-benzoDBT

MATERIALS AND METHODS

Medium and cultivation. *R. erythropolis* H-2 was grown in medium A in Section 1 of this chapter with DBT or its derivatives as a sole sulfur source. Cultivation was done at 30°C in test-tubes (ϕ=18 mm) containing 5 ml medium with reciprocal shaking at 300 rpm. For resting cells reaction the strain was cultivated at 30°C for 2 days in 2-l flasks containing 500 ml medium with reciprocal shaking at 100 rpm. DBT was added to the medium as a sulfur source at 50 mg/l. Cells were harvested at 4°C by centrifugation at 10,000 x *g* for 15 min, washed once with 0.85% NaCl and kept at -20°C.

Resting cells reaction. Frozen cells were lyophilized and then used for the resting cells reaction. The reaction mixture contained 100 μl of 1 M potassium phosphate buffer (pH 7.0), 400 μl of *n*-tetradecane, 40 mg of lyophilized cells and 1 mM each DBT derivatives in a final volume of 1 ml. The reaction proceeded in test-tubes (ϕ=18 mm) at 30°C with reciprocal shaking (300 rpm).

Analyses and chemicals. DBT and its derivatives were determined by gas chromatography or high-performance liquid chromatography as described in Section 1 of this chapter. 3,4-Benzodibenzothiophene and 2,8-dimethyldibenzothiophene were purchased from Aldrich Chem. Co., Milwaukee, U.S.A. and Tokyo Kasei Kogyo Co., Tokyo, Japan, respectively. 4,6-Dimethyldibenzothiophene was synthesized from DBT as described [55] All other reagent were of analytical grade, commercially available, and used without further purification.

RESULTS AND DISCUSSION

Growth of *R. erythropolis* H-2 in the medium containing DBT derivatives

The strain was cultivated in medium A with DBT or its derivatives as sole sources of sulfur at 50 mg/l. As shown in Table I, this strain grew more or less on four aromatic sulfur compounds tested. Though 3,4-benzoDBT was not a good sulfur source for this strain, two dimethyl DBTs as well as DBT could support the growth of this strain well.

Table I. Growth in the Presence of DBT Derivatives as Sulfur Source
The strain was cultivated in medium A with 50 mg/l DBT derivatives in test-tube for 4 days.

Compound	Growth (A_{660})	pH
DBT	5.7	4.0
2,8-dimethyl DBT	4.7	4.9
4,6-dimethyl DBT	5.0	4.5
3,4-benzoDBT	1.7	5.6

Degradation of DBT derivatives in the presence of hydrocarbon by resting cells system

The resting cells reaction was done with adding DBT derivatives at 1 mM in the presence of *n*-tetradecane. As shown in Fig. 2, DBT and 2,8-dimethyl DBT were degraded in a similar manner. The degradation rate of 4,6-dimethyl DBT was approximately one-half that of DBT. These DBT related compounds were degraded completely within 6 h. The results suggest that this strain's enzyme system involved in DBT desulfurization could act on dimethyl substituted DBT as well as DBT itself; that is, there is no steric hindrance between methyl groups and this enzyme system. Considering this strain also has the efficient desulfurization activity in the presence of hydrocarbon, it is

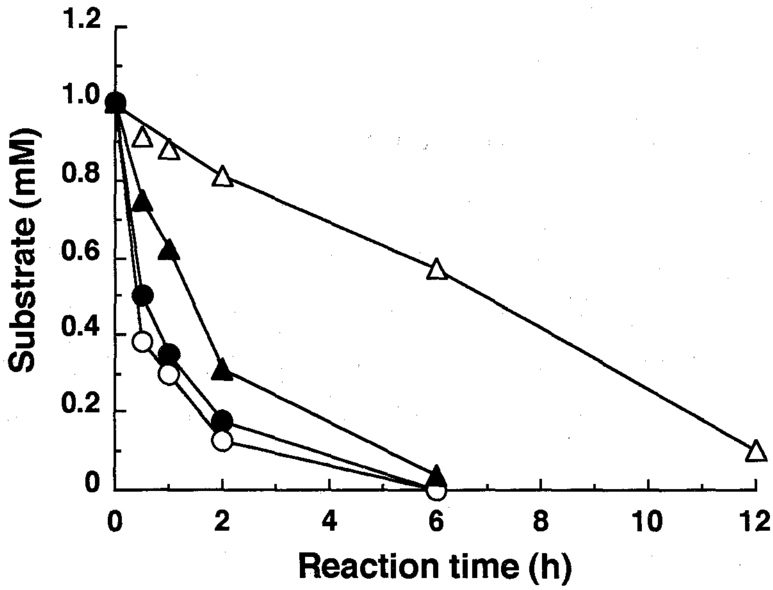


Fig. 2. Degradation of DBT Derivatives in the Presence of Hydrocarbon by Resting Cells System.

The reaction mixture contained 100 μmol potassium phosphate buffer (pH 7.0), 40 mg lyophilized cells of *R. erythropolis* H-2, 400 μl *n*-tetradecane and 1 μmol DBT (●), 2,8-dimethyl DBT (○), 4,6-dimethyl DBT (▲), and 3,4-benzoDBT (△) in a total volume of 1 ml. Other reaction conditions are described in text.

evident that these properties of this strain is significantly favorable for the practical desulfurization of petroleum. On the contrary, 3,4-benzoDBT was degraded very slowly, the amount of the substrate reduced to 0.1 mM after 12 h (Fig. 2). The enzyme system might not utilize well such a compound containing one more benzene ring.

The product in the reaction mixture using 4,6-dimethyl DBT as a substrate was analyzed by gas chromatograph-mass spectrometer. As a result, the expected mass ion at m/z 198 which corresponds to the molecular weight of monohydroxy 4,6-dimethyl DBT was clearly detected. In other DBT derivatives, the expected mass ions at m/z 198, 220 were obtained, respectively. Therefore it was thought that the microbial desulfurization of these DBT derivatives occurred like DBT, hydroxylated biphenyls were formed.

SUMMARY

R. erythropolis H-2 can grow on 2,8-dimethyl DBT and 4,6-dimethyl DBT. In the resting cells reaction, these DBT derivatives added at 1 mM were completely degraded within 6 h as well as DBT. The reaction products were assumed to be the monohydroxy biphenyl form of corresponding DBT derivatives.

CHAPTER II
Enzyme System Involved in Dibenzothiophene Desulfurization

Section 1. Enzymatic Desulfurization of Dibenzothiophene by a Cell-free System of *Rhodococcus erythropolis* D-1

Microbial desulfurization of DBT without loss of carbon has been demonstrated as shown in the Chapter I. The intermediates in the microbial degradation of DBT by *R. rhodochrous* IGTS8 have also been elucidated [45], and the genes involved in DBT degradation by this strain have been identified [48]. However, enzymatic conversion of DBT to 2-HBP has never been reported. As described in Section 1 of Chapter I, I isolated *R. erythropolis* D-1 which utilizes DBT as a sole source of sulfur and demonstrated the efficient desulfurization of DBT to 2-HBP by both growing and resting cells of this strain. In this section, I describe the enzymatic desulfurization of DBT.

MATERIALS AND METHODS

Cultural conditions. The medium for the cultivation, medium A, was as described in Section 1 of Chapter I. *R. erythropolis* D-1 was grown in 500 ml of medium A containing DBT at 0.136 mM in a 2-liter shaking flask. DBT dissolved in ethanol was added to sterilized medium A. Cultivation proceeded at 30°C for 2 days with reciprocal shaking (100 strokes/min). Cells were harvested at 4°C by centrifugation at 10,000 x *g* for 15 min, washed once with 0.85 % NaCl and stored at -20°C before use.

Preparation of cell-free extract. All operations were carried out below 15°C. Fifty grams (wet weight) of frozen cells were resuspended in 100 ml of 100 mM potassium phosphate buffer (pH 7.0), then disrupted with an ultrasonic oscillator (20 kHz). The cell debris was removed by centrifugation at 12,000 x *g* for 30 min. The supernatant solution was used as the cell-free extract.

Enzyme assays. DBT-degrading activity was assayed by measuring the amount of decreasing DBT by means of gas chromatography (GC) or high performance liquid chromatography (HPLC). The standard reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 0.1 mM DBT, 1 mM NADH and a suitable amount of enzyme solution in a total volume of 0.5 ml. The reaction was initiated by adding DBT dissolved in 9 μl of ethanol, and proceeded at 30°C with shaking (300 strokes/min). After an appropriate period (typically 30 to 60 min), 50 μl of 1 N HCl was added to terminate the reaction. Thereafter, 400 μl of ethyl acetate was added to extract DBT, and a portion of the ethyl acetate layer was removed and centrifuged. The supernatant was analyzed by GC or HPLC. The enzyme activity was expressed as the amount of protein that degraded 1 nmol of DBT per min at 30°C.

Analytical methods. GC and HPLC were performed as described in Section 1 of Chapter I. The protein concentration was determined by the method of Bradford [56] with bovine serum albumin as the standard.

Chemicals. NADH disodium salt, NAD^+, NADPH tetrasodium salt, and $NADP^+$ were gifts from Kohjin Co., Ltd., Tokyo, Japan, and 5,6,7,8-tetrahydrobiopterin was a gift from Suntory Co., Osaka, Japan. All other chemicals were of reagent grade and were used without further purification.

RESULTS AND DISCUSSION

When DBT degradation was tested using cell-free extracts of *R. erythropolis* D-1, the reaction proceeded so slowly that it took 9 h to degrade 0.1 mM DBT (data not shown). After the cell-free extract was dialyzed overnight against three changes of a total volume of 12 liters of 100 mM potassium phosphate buffer (pH 7.0), the activity was no longer detectable in the dialyzed cell-free extract. Several well-known cofactors of oxido-reductases were examined to determine whether they could restore the activity (Table I). NADH was effective, whereas NADPH could not substitute for

Table I. Requirements for DBT Degrading Activity in Cell-free Extract

The reaction was done using the dialyzed cell-free extract. The reaction mixtures contained 2.5 mg of protein. NAD^+, NADH, $NADP^+$, and NADPH were added at 500 nmol. FAD, FMN, PQQ, and H_4BP were added at 50 nmol.

Cofactor	Activity (nmol/min)
None	0
NAD^+	0.6
NADH	1.9
$NADP^+$	0
NADPH	0.2
FAD	0
FMN	0
Cytochrome *c*	0
PQQ	0
H_4BP[a]	0

[a] 5,6,7,8-Tetrahydrobiopterin

NADH. The reaction proceeded in the presence of NAD^+ as shown in Table I. It is speculated that the reaction might involve a reduction followed by an oxidation or vice versa. No other cofactors were required for DBT degradation. The amount of DBT linearly decreased with the reaction time (Fig. 1). There was clear difference between the decreasing rate of DBT and the increasing rate of 2-HBP. Nevertheless, DBT was quantitatively converted to 2-HBP in this cell-free system. As shown in Fig. 2, the formation of 2-HBP continued until the stoichiometrical point after DBT disappeared completely. This suggested that intermediates from DBT to 2-HBP were temporarily accumulated. These intermediates remain to be identified yet.

As shown in Fig. 3 the activity was enhanced by increasing the protein concentration when DBT was added at 0.1 mM. Furthermore, the reaction rate was stimulated by increasing the NADH concentration up to 5 mM (Fig. 4). The relation between the NADH concentration and the reaction rate followed Michaelis-Menten kinetics. The above data confirmed the enzymatic desulfurization of DBT in the cell-free system for the first time.

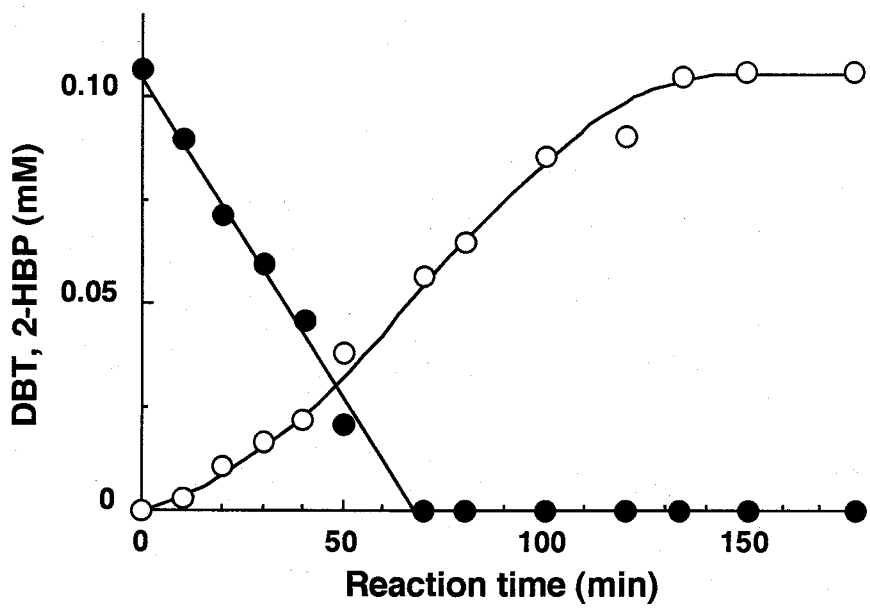


Fig. 1. Time Course of Enzymatic DBT Desulfurization to 2-HBP by the Cell-free Extracts.

The protein concentration was 3.6 mg/ml. Other reaction conditions are described in Materials and Methods. ●, DBT; O, 2-HBP.

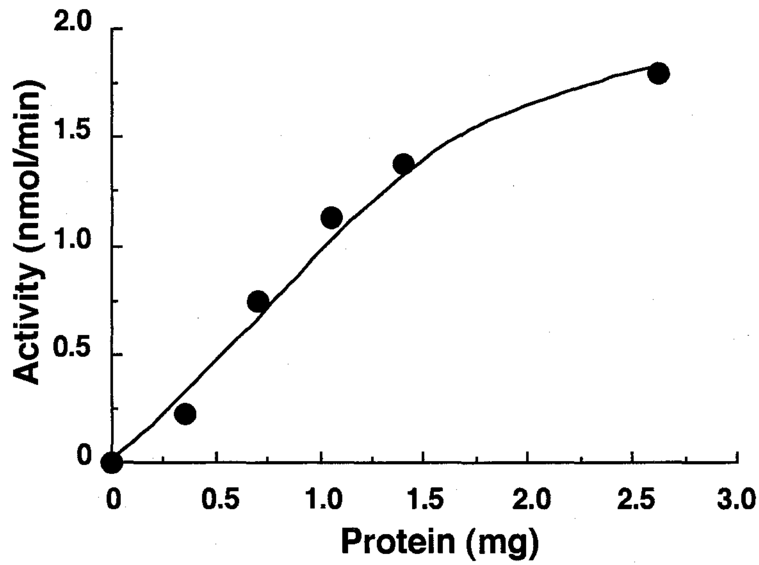


Fig. 2. DBT Degradation by Various Concentrations of the Cell-free Extracts.

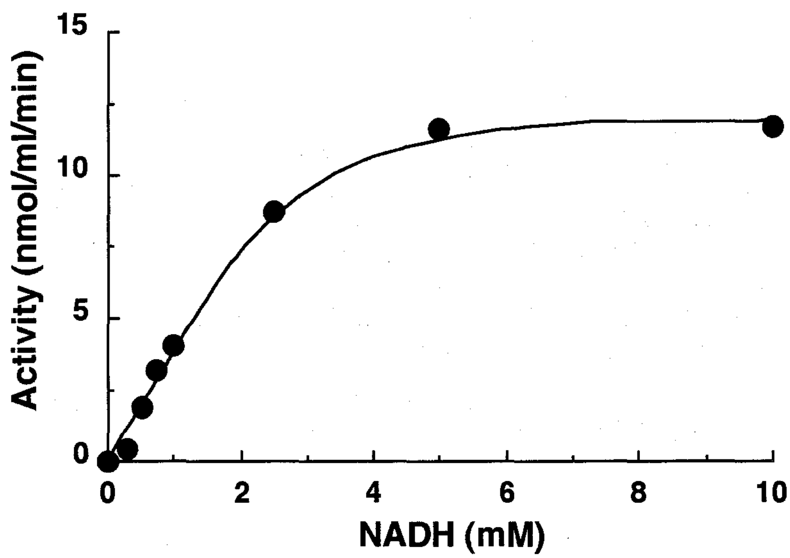


Fig. 3. Effect of NADH Concentration on DBT Degradation.
The reaction mixture contained 1.8 mg of protein.

Little information is available regarding the degradation of aromatic sulfur compounds. Monticello *et al.* found that DBT-degrading activity was present in a cell-free extract of *Pseudomonas* sp. grown on naphthalene [37]. However, the major product from DBT was identified as the hemiacetal form of 4-[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid, which still retains the sulfur atom.

Some recently isolated organisms can remove sulfur from DBT without destroying the carbon skeleton, and these have been considered attractive for microbial desulfurization. However, no information is available concerning the enzymes of these strains for DBT degradation. This is the first report to our knowledge that describes an enzyme system catalyzing the conversion of DBT to 2-HBP. I proved that NADH is essential for DBT desulfurization in this cell-free system. The conversion of DBT to 2-HBP is considered to proceed by a multienzyme system judging from the possible intermediates previously proposed [42, 48]. However it will remain unclear at which step(s) in the conversion NADH is required, until each enzyme is highly purified.

SUMMARY

The enzymatic desulfurization of DBT to 2-HBP was detected for the first time in cell-free extracts of *R. erythropolis* D-1 grown on DBT as a sole source of sulfur. In the dialyzed cell-free extracts, NADH was absolutely required for the activity. DBT desulfurization proceeded linearly with the reaction time and stoichiometric amounts of 2-HBP were finally formed . The activity was stimulated by increasing the protein and NADH concentrations up to 5 mg/ml and 5 mM, respectively, when 0.1 mM DBT was present in the reaction mixture.

Section 2. Involvement of Flavin Coenzyme in Dibenzothiophene Degrading Enzyme System from *Rhodococcus erythropolis* D-1

In the desulfurization process from DBT to 2-HBP, two carbon-sulfur bonds must be cleaved. Some enzyme systems involved in the cleavage of carbon-sulfur bond of aromatic sulfonate have been characterized. For example, sulfonobenzoate was converted to protocatechuate by the purified enzymes from *Comamonas testosteroni* T-2 [57], and benzenesulfonate and *p*-toluenesulfonate were converted to catechol and 4-methylcatechol by that from *Alcaligenes* sp. O-1 [58].

In Section 1 of this chapter, I showed that DBT was enzymatically desulfurized by cell-free extracts of *R. erythropolis* D-1 and that NADH was required for the activity. Denome *et al.* demonstrated that DBT degrading activity in the cell lysates was increased in the presence of FAD [59]. However, no detailed analyses have been done such as measurement of the product and the remaining substrate, and observation of the course of DBT degradation. In this section, I describe the involvement of a flavin coenzyme, FAD or FMN, in the enzymatic DBT degradation and their inhibition of the activity at high concentrations of the coenzymes.

MATERIALS AND METHODS

The strain *R. erythropolis* D-1 was cultivated as described in Section of Chapter 1. The harvested cells were suspended in 50 mM Tris-HCl buffer (pH 8.0), then disrupted with an ultrasonic oscillator (20 kHz). The cell debris was removed by centrifugation at 12,000 x *g* for 30 min. Solid ammonium sulfate was added to this supernatant to give 30% saturation. After being stirred for 2 h, the precipitate was removed by centrifugation (12,000 x *g*, 30 min) and the supernatant was further saturated with ammonium sulfate to 60% saturation. After 2 h the suspension was centrifuged at 12,000 x *g* for 30 min, and the resulting pellet was dissolved in a minimum volume of the buffer and then dialyzed overnight against the same buffer. The dialyzed enzyme solution was put on a DEAE-Sepharose Fast Flow column (2.7 x 28 cm, Pharmacia LKB, Uppsala, Sweden), which had been equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The column was washed well with the same buffer and the adsorbed enzyme was eluted with a linear gradient of KCl of from 0 to 1 M in the same buffer. Each protein peak was concentrated and desalted by an ultrafiltration filter (YM-10, Amicon, Inc., Beverly, U. S. A.) and measured for the enzyme activity.

DBT-degrading activity was determined by measuring the amount of decreasing DBT by gas chromatography and high performance liquid chromatography as described in Section 1 of Chapter I with a slight modification. The standard reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 0.1 mM DBT, 1 mM NADH, and a suitable amount of enzyme solution in a total volume of 0.5 ml. The reaction was done at 35°C with shaking (300 strokes per min). The enzyme activity was expressed as the amount of protein that degraded 1 nmol of DBT per min at 35°C. The protein concentration was measured by the method of Bradford [56]. Mass spectra were taken on a Perkin Elmer Q-Mass 910 gas chromatograph mass spectrometer with a DB 17 capillary column (0.25 mm x 30 m, J&W Scientific, Folsom, U. S. A.). FAD and FMN were purchased from Nakalai Tesque, Kyoto, Japan. All other chemicals were of analytical grade, commercially available.

RESULTS AND DISCUSSION

The specific activity of DBT-degrading enzyme in cell-free extracts was 0.8 nmol/min/mg-protein and after ammonium sulfate fractionation it was 3.4 nmol/min/mg-protein. However, after DEAE-Sepharose Fast Flow chromatography it was 2.2 nmol/min/mg-protein; this purification step evidently lead to the decrease of the specific activity. Though we had examined the effects of several well-known cofactors of oxidoreductases for

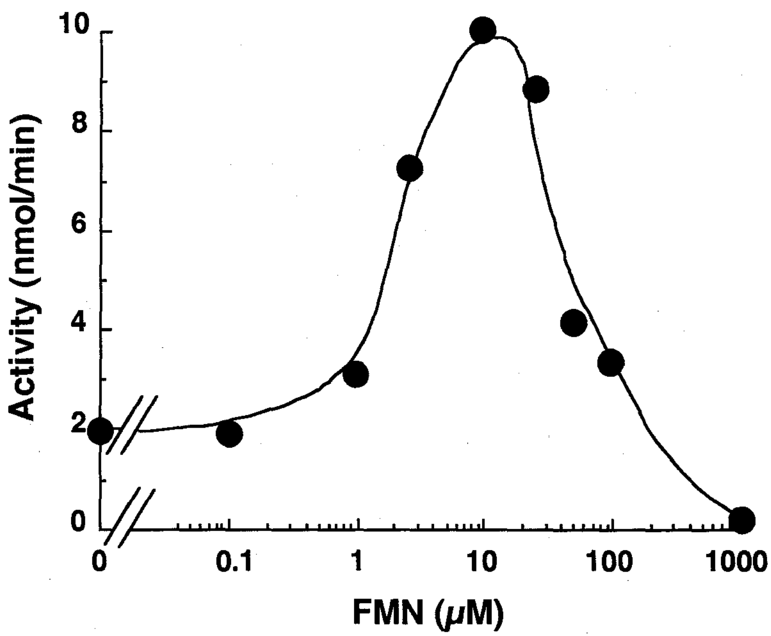


Fig. 1. Effects of FMN on the Activity.
The reaction was done using the enzyme solution purified by DEAE-Sepharose column chromatography. The reaction mixture contained 0.5 mg of protein, 3 mM NADH, 0.1 mM DBT, indicated concentrations of flavin coenzyme, and 50 mM potassium phosphate buffer (pH 7.0). Other reaction conditions are described in text.

DBT-degrading activity, these compounds were reexamined in the presence of NADH using the enzyme preparation purified by the anion exchange chromatography. It became apparent that the addition of FMN or FAD enhanced the activity. Under these experimental conditions; the optimal concentration of FMN was 10 μM, at which the specific activity was elevated about five-fold compared with the case of no addition of FMN (Fig. 1). It has been reported that some flavoproteins such as NADH-ferredoxin$_{NAP}$ reductase [60], putidaredoxin [61], and NADH-ferredoxin$_{TOL}$ reductase [62], contained loosely bound flavin cofactors, which were lost during purification. In this respect, it was thought that a part of flavin coenzyme was removed from the DBT-degrading enzyme system during DEAE-Sepharose column. Furthermore, an excess of FMN was inhibitory for DBT degradation; no activity was detected upon adding 1 mM FMN. The same result was obtained in the case of FAD (Fig. 2). On the contrary, when the enzymatic reaction was done by

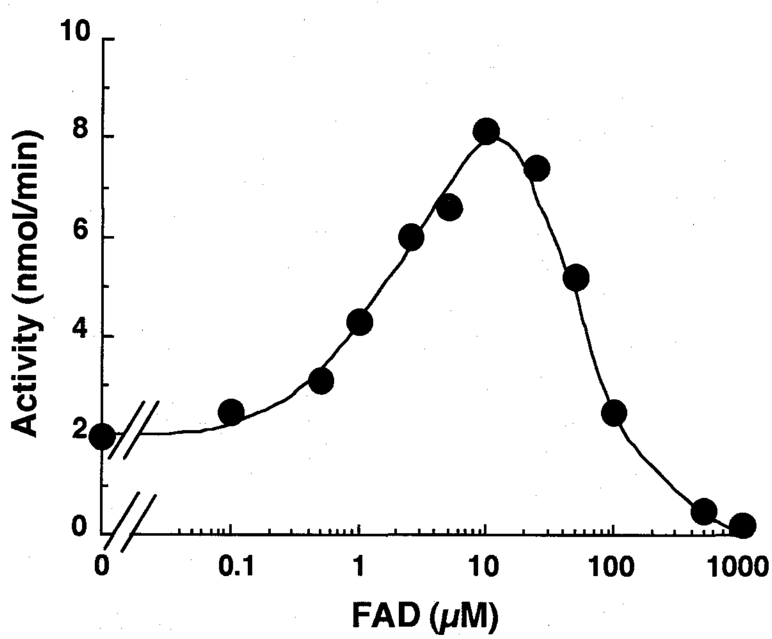


Fig. 2. Effects of FAD on the Activity.
The reaction was done using the enzyme solution purified by DEAE-Sepharose column chromatography. The reaction mixture contained 0.5 mg, 3 mM NADH, 0.1 mM DBT, indicated concentrations of flavin coenzyme, and 50 mM potassium phosphate buffer (pH 7.0). Other reaction conditions are described in text.

using the enzyme preparation fractionated by ammonium sulfate or the cell-free extracts, flavin coenzyme did not stimulate the activity and only an inhibitory effect was observed (Fig. 3).

The enzymatic reaction where 10 μM FMN was added to the standard reaction mixture was done using the enzyme preparation purified by DEAE-Sepharose column chromatography, then it has become evident that DBT sulfone was a product from DBT though 2-HBP was the final metabolite from DBT when using the ammonium sulfate fractionated enzyme preparation. As shown in Fig. 4, the reaction from DBT to DBT sulfone proceeded stoichiometrically. The product in the reaction mixture was also identified as DBT sulfone by gas chromatograph-mass spectrometer. In this work, 2-HBP formation was not detectable at all even if all the fractions eluted from DEAE

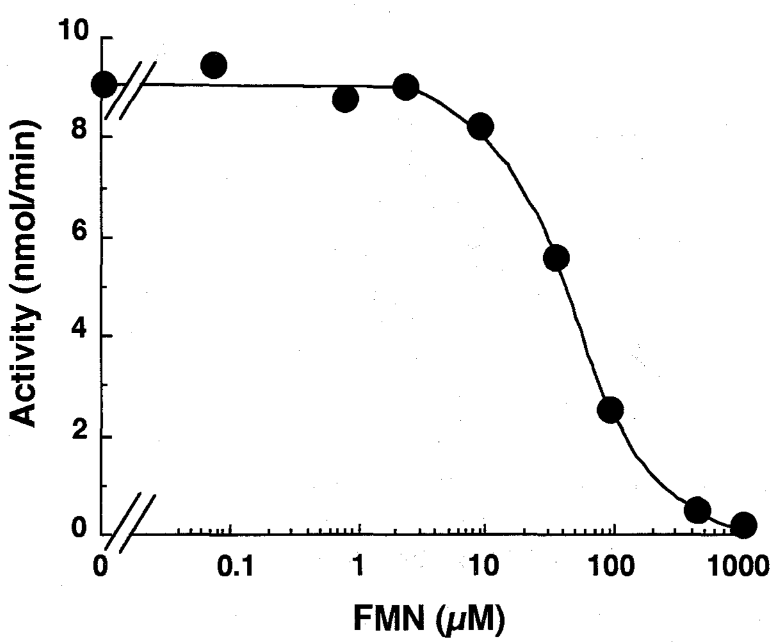


Fig. 3. Effects of FMN on the Activity of the Enzyme Fractionated by Ammonium Sulfate.

The reaction was done using the enzyme fractionated by ammonium sulfate. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 3 mg of protein, 3 mM NADH, 0.1 mM DBT and indicated concentrations of flavin coenzyme. Other reaction conditions are described in text.

column chromatography were combined. This loss of the 2-HBP forming activity in the combined fractions might be due to the enzyme(s) instability during the purification or the adsorption of the enzyme(s) involved in the conversion of DBT sulfone to 2-HBP on the DEAE-Sepharose column.

I have shown that NADH was necessary for the enzyme activity in the previous section and the reaction rate as a function of the NADH concentration followed Michaelis-Menten kinetics. However, that as a function of the FMN concentration did not follow it and a high concentration of FMN inhibited the activity. In this study the concentration of FMN required for the maximal enzymatic activity (10 μM) was significantly lower than that of NADH (5 mM). It appeared that FMN acted catalytically and NADH did as a substrate. It has been known that some oxygenases have a noncovalently bound flavin

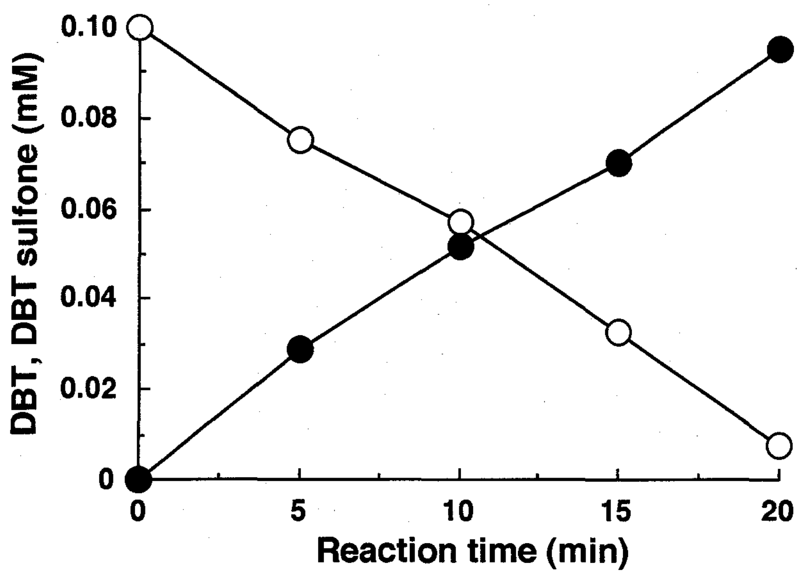


Fig. 4. Course of the Enzymatic Conversion of DBT to DBT Sulfone.
The reaction was done by adding 10 μM FMN using the enzyme solution purified by DEAE-Sepharose column chromatography. The reaction mixture contained 0.5 mg of protein. Other reaction conditions are described in text. ○, DBT; ●, DBT sulfone.

coenzyme as a prosthetic group and require NAD(P)H as a reductant [58, 60-63]. The enzyme involved in the conversion of DBT to DBT sulfone seems to be a kind of oxygenase. Thus, I found not only stimulatory effects of FAD and FMN, but also their inhibitory effects on the activity at high concentrations. These findings led me to suggest that these coenzymes might be important in the regulation of the enzyme system of DBT degradation.

SUMMARY

In the process of the purification of a DBT-degrading enzyme system from cell-free extracts of *R. erythropolis* D-1, flavin coenzymes, FMN and FAD, were found to be involved in the enzymatic degradation of DBT in addition to NADH. Under these experimental conditions, the optimal concentrations of FMN and FAD were both 10 μM and the activity was completely inhibited by adding 1 mM FMN or FAD. DBT was converted to DBT sulfone stoichiometrically and 2-HBP formation was not observed when the reaction was done using the enzyme preparation purified by DEAE-Sepharose column chromatography.

Section 3. Regulation of Dibenzothiophene Degrading Enzyme Activity of *Rhodococcus erythropolis* D-1

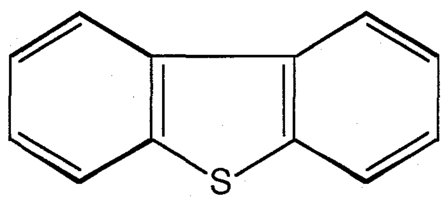
Recently, it was reported that DBT was not degraded by the resting cells of *Rhodococcus* sp. strain SY1 grown in the medium containing sulfate [64]. However, there is no enzymatic evidence available for the regulation of the DBT degrading enzyme system yet. Herein, in this section, I examined the effects of various sulfur compounds including DBT and its metabolites and analogs on the activity of the DBT degrading enzyme of *R. erythropolis* D-1.

MATERIALS AND METHODS

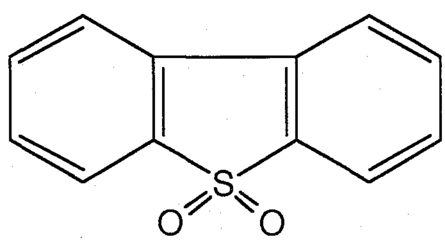
Culture conditions. The basal medium used was medium A as described in Section 1 of chapter I. *R. erythropolis* D-1 was grown in 500 ml of medium A containing various sulfur compounds in a 2-l shaking flask or 5 ml of the same medium in a test tube (18 by 180 mm). Cultivation was done at 30°C for 2 days with reciprocal shaking (100 strokes per min for 2-l flasks and 300 strokes per min for test tubes).

Preparation of cell-free extracts. All operations were done below 15°C. Cells were collected by centrifugation at 10,000 x *g* for 15 min, washed once with 0.85% NaCl. Ten grams (wet weight) of cells were suspended in 20 ml of 50 mM Tris-HCl buffer (pH 8.0), then disrupted with an ultrasonic oscillation (20 kHz). The cell debris was removed by centrifugation at 12,000 x *g* for 30 min. The supernatant was thoroughly dialyzed against the same buffer. The dialyzed solution was referred to as cell-free extracts and was used as the enzyme.

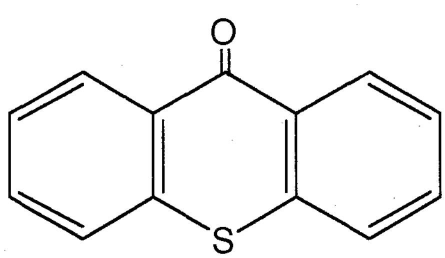
Enzyme assay. The assay of DBT-degrading activity was performed by measuring the amount of decrease in DBT concentration by HPLC as described in Section 1 of this Chapter with a slight modification. The standard reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 0.1 mM DBT, 3 mM NADH and a suitable amount of enzyme solution in a total volume of 0.5 ml. The enzyme reaction was done at 35°C with shaking (300 strokes per min). The enzyme activity was expressed as the amount of protein which degraded 1 nmol of DBT per min. The protein concentration was determined by the method of Bradford [56] with bovine serum albumin as the standard.



I



II



III

Fig. 1. Chemical Structure of DBT and its Analogs.
(I) DBT, (II) DBT sulfone, (III) Thioxanthen-9-one

Chemicals. DBT sulfone and thioxanthen-9-one were obtained from Aldrich, Milwaukee, Wis., U.S.A. 2,2'-Dihydroxybiphenyl (DHBP) were purchased from Tokyo Kasei Co., Tokyo, Japan. Dimethyl sulfone, methanesulfonic acid and biphenyl were obtained from Nakalai Tesque, Kyoto, Japan. All other reagents were of analytical grade and commercially available.

RESULTS

Effects of sulfur compounds on the enzyme production

As reported in Section 1 of Chapter I, this strain grew on several sulfur compounds other than DBT as a sole source of sulfur. The strain was cultivated in medium A with 0.3 mM of six such sulfur compounds as sole sources of sulfur, and the DBT-degrading activity was measured in cell-free extracts prepared from the harvested cells. As shown in Table I, significant enzyme activities were found when the strain was grown with DBT alone and its analogs, thioxanthen-9-one or DBT sulfone, but not with inorganic sulfate, methanesulfonic acid or dimethyl sulfone. Growth levels of the strain were the same using each of these compounds as the sulfur source. Next, the strain was grown in medium A containing both 0.3 mM DBT and 0.1 mM of each

Table I. Effects of Sulfur Compounds on the Specific Enzyme Activity
Cells were cultivated in medium A with each sulfur compound at 0.3 mM. The enzyme activities in the cell-free extracts were measured as described in text.

Sulfur compounds	Growth (OD_{660})	Specific activity (nmol/min/mg)
DBT	3.9	1.9
Sodium sulfate	3.0	0
Methanesulfonic acid	3.5	0
Dimethyl sulfone	2.6	0
Thioxanthen-9-one	3.1	1.6
DBT sulfone	4.3	1.5

sulfur compound tested in Table I, and the enzyme activities were assayed. All the sulfur compounds tested decreased the specific activities of the enzyme to 6-63% (Table II). By contrast, the growth levels were similar. Among the sulfur compounds the inhibitions by methanesulfonic acid and sodium sulfate were significant. The addition of various concentrations of methanesulfonic acid and sodium sulfate to the medium including 0.3 mM DBT revealed that 0.1 mM methanesulfonic acid and 0.5 mM sodium sulfate completely repressed the specific enzyme activity (Fig. 2).

Effects of sulfate, 2-HBP and its analogs on the DBT degradation by cell-free extracts

I examined the effects of the DBT desulfurization metabolites, sulfate and 2-HBP, on the enzyme activity. In addition, I also tested the effects of 2-HBP analogs, DHBP and biphenyl. These compounds were added to the reaction mixture containing the cell-free extracts prepared from cells grown with DBT as a sole source of sulfur. 2-HBP and DHBP markedly inhibited the enzyme activity (80% and 70% inhibition at 1 mM, respectively), whereas sulfate and biphenyl hardly inhibited the activity even at 1 mM (Fig. 3).

Table II. Effects of Sulfur Compounds Addition to Medium Containing DBT on the Specific Enzyme Activity

Cells were cultivated in medium A with 0.3 mM DBT and each sulfur compound at 0.1 mM. The enzyme activities in the cell-free extracts were measured as described in Materials and Methods.

Sulfur compounds	Growth (OD_{660})	Specific activity (nmol/min/mg)
DBT	3.7	1.9
DBT+Sodium sulfate	3.9	0.53
DBT+Methanesulfonic acid	4.5	0.12
DBT+Dimethyl sulfone	4.0	1.2
DBT+Thioxanthen-9-one	4.4	1.0
DBT+DBT sulfone	4.2	1.2

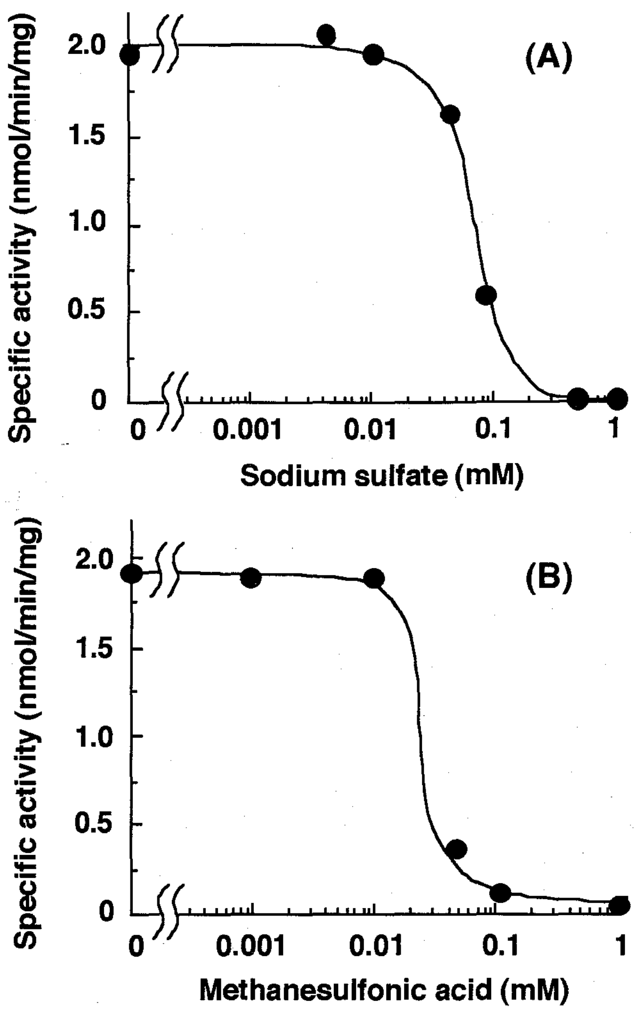


Fig. 2. Enzyme Activities after Growth with Various Concentrations of Sodium Sulfate and Methanesulfonic Acid in the Presence of DBT.

Cells were cultivated in medium A with 0.3 mM DBT in the presence of the indicated concentrations of sodium sulfate (A) or methanesulfonic acid (B). The enzyme activities in the cell-free extracts were measured as described in Materials and Methods.

Effects of 2-HBP and its analogs on the growth

Strain D-1 was cultivated for 2 days in medium A containing 0.3 mM DBT or sodium sulfate supplemented with three biphenyl derivatives. As Fig. 4

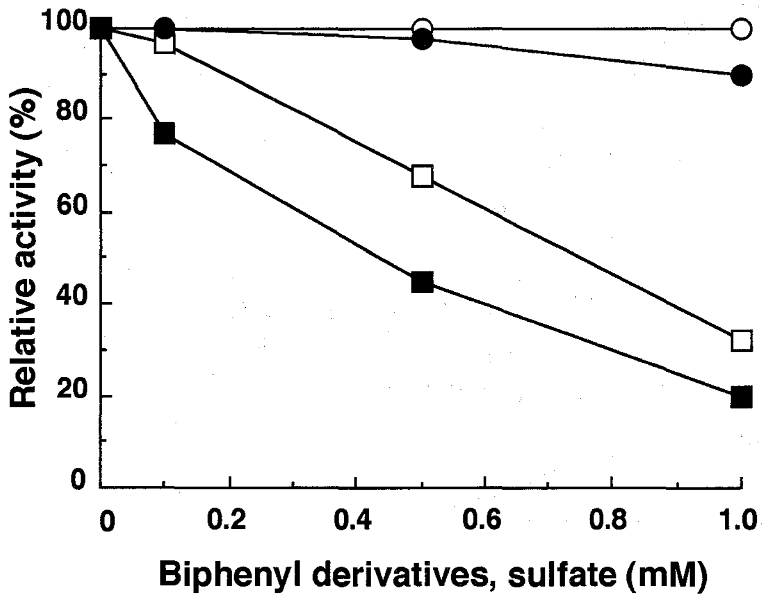


Fig. 3. DBT Degradation by Cell-Free Extracts in the Reaction Mixtures with Various Concentrations of 2-HBP, DHBP, Biphenyl and Sulfate.

The cell-free extract prepared from the cells grown in the medium with DBT as a sole source of sulfur was used. The enzyme reaction was done in the standard reaction mixture with various concentrations of 2-HBP (■), DHBP (□), biphenyl (●), and sodium sulfate (○).

shows, 2-HBP and DHBP inhibited the growth of this strain. 2-HBP was more inhibitory than DHBP. Biphenyl showed little inhibition against the growth. In all cases, longer incubation resulted in no further growth. These findings are similar to those shown in Fig. 3. Growth inhibition by 2-HBP and DHBP was greater in the medium with DBT as a sulfur source than in the medium with sodium sulfate (Fig. 4A, B) .

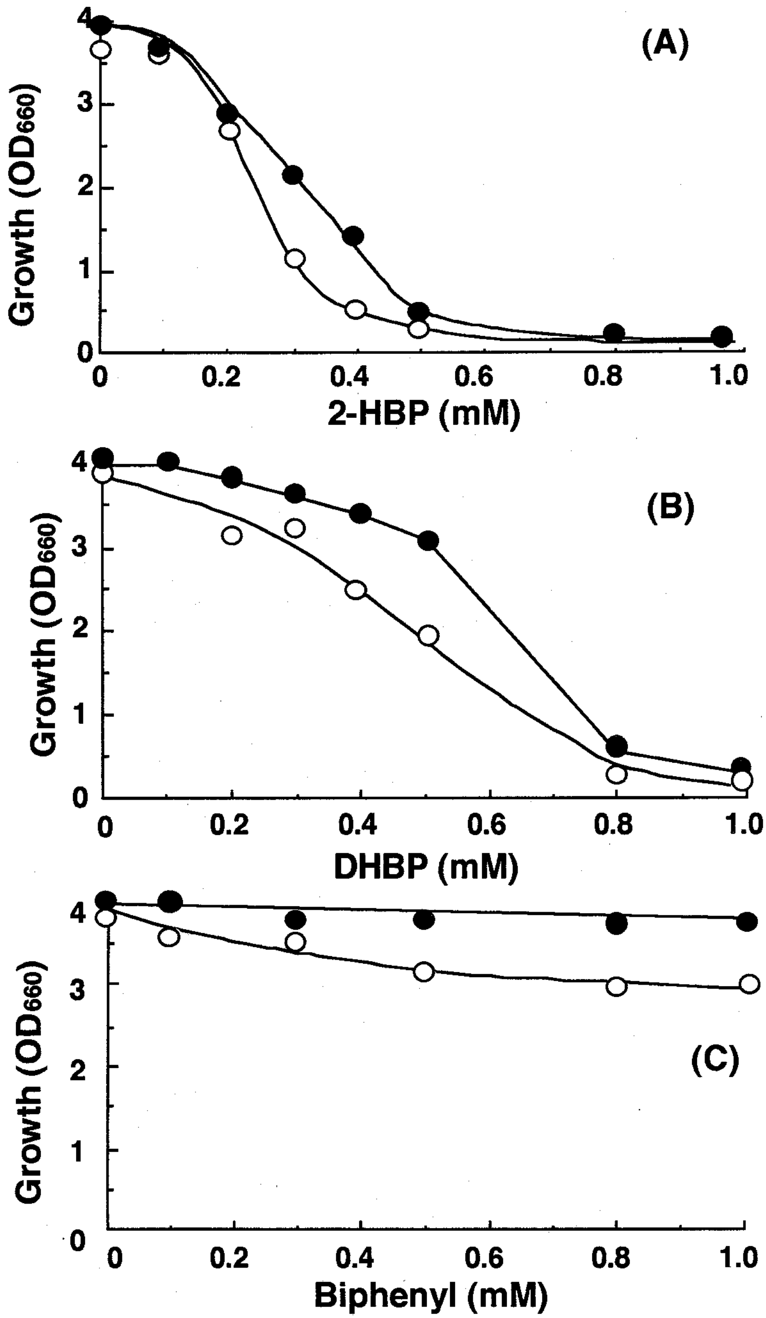


Fig. 4. Effects of 2-HBP, DHBP and Biphenyl Concentrations on the Cell Growth.

Cells were cultivated in medium A with various concentrations of 2-HBP (A), DHBP (B), and biphenyl (C) in the presence of 0.3 mM DBT (○) or 0.3 mM sodium sulfate (●).

DISCUSSION

2-HBP and DHBP seemed to be intrinsically toxic to cells of *R. erythropolis* D-1 from the results of growth inhibition by these compounds at 0.5 mM and 1 mM, respectively (Fig. 4). However, at lower concentrations both biphenyl derivatives were more inhibitory in the medium with DBT than in that with sulfate. This could be explained by the fact that they inhibited the DBT degrading enzyme system as shown in Fig. 3; that is, the inhibition may have interrupted the sulfur supply required for the cell growth. While both 2-HBP and DHBP at 1 mM inhibited the cell growth and the DBT degrading enzyme system, biphenyl had no inhibitory effects on the growth and the enzyme activity. It can therefore be presumed that the hydroxy groups attached to the biphenyl nucleus play an important role in the growth and enzymatic inhibition.

The rate of synthesis of arylsulfatase has been demonstrated to vary with the type of sulfur source [65]. Arylsulfatase is a kind of esterase which hydrolyzes aromatic sulfate ester to the corresponding phenol and inorganic sulfate. This was induced under the sulfate limitation in *Escherichia coli* [66], *Pseudomonas putida* [66] and *Staphylococcus aureus* [66]. By contrast, it was repressed by sulfate in *P. aeruginosa* [67] and *Klebsiella aerogenes* [68-70]. A negative regulator gene for the synthesis of arylsulfatase in *K. aerogenes* was cloned, and the gene was demonstrated to code for a functional dihydrofolate reductase protein [71]. Other enzyme systems involved in the cleavage of carbon-sulfur bonds have been reported in *Alcaligenes* sp. O-1 [58] and *Comamonas testosteroni* [57]. Nevertheless, there is no information about the regulation of the production of these enzymes.

In various works previously reported concerning microbial DBT desulfurization, DBT has been added to the synthetic medium as a sole source of sulfur. Here I presented the possibility of the induction and repression of the DBT degrading enzyme system using the cell-free system of *R. erythropolis* D-1. Although the enzyme production may be induced by DBT and its analogs, thioxanthen-9-one and DBT sulfone, it may be repressed by organic or inorganic sulfur even in the presence of DBT. This finding suggests that sulfate was released enzymatically from each sulfur compound and the excess sulfate repressed the enzyme production.

SUMMARY

The regulation of DBT degrading activity of *R. erythropolis* D-1 was examined. The enzymatic activity involved in DBT degradation of the strain D-1 was found in cell-free extracts of cells grown not only with DBT as a sole sulfur source but also with its analogs, thioxanthen-9-one and DBT sulfone. The activity was completely repressed in a medium with 0.5 mM sodium sulfate or 0.1 mM methanesulfonic acid even in the presence of DBT. The enzyme activity in the cell-free extracts of this strain was inhibited by a degradation product, 2-HBP, and its analog, DHBP, but not by sodium sulfate and biphenyl. 2-HBP and DHBP also significantly inhibited the growth of this strain when it was cultivated in the medium supplemented with DBT as a sole source of sulfur.

Section 4. Purification and Characterization of Dibenzothiophene Oxygenase from *Rhodococcus erythropolis* D-1

It has been established that three genes designated *soxABC* were required for DBT desulfurization by *Rhodococcus* sp. IGTS8 [59]. Each *sox* gene was expressed in *Escherichia coli* under control of the inducible promoter. By using the cell-free extracts from the *E. coli* overexpressing each of the Sox proteins, it has been assigned their functions that SoxA is a protein responsible for metabolizing DBT sulfone to an unidentified intermediate, SoxB is a protein converting this unknown intermediate into 2-HBP, and SoxC is a protein that oxidizes DBT to DBT sulfone. However, neither these reactions involved in DBT desulfurization has yet been confirmed by the pure enzyme protein(s).

In section 1 of this chapter, I revealed that DBT was enzymatically desulfurized to 2-HBP by cell-free extracts of *R. erythropolis* D-1 and that NADH was required for the activity. Moreover, I presented that flavin coenzyme involved in DBT desulfurizing activity that DBT was converted to DBT sulfone not to 2-HBP by the enzyme preparation purified by DEAE-Sepharose column chromatography in section 2 of this chapter. It has also been demonstrated that DBT degrading activity, SoxC activity, in the cell lysates was increased in the presence of FAD and that SoxC should be so closely related to the family of acyl-CoA dehydrogenases on the basis of the similarity of amino acids sequences [59]. Some of acyl-CoA dehydrogenases bind FAD noncovalently, FAD has been shown to increase the enzymatic activity for such acyl-CoA dehydrogenases [72, 73].

In this section, I found out that the reaction from DBT to DBT sulfone was catalyzed by muticomponent enzymes and now describe the purification and some properties of a component B of DBT oxygenase system.

MATERIALS AND METHODS

Growth of microorganisms and the preparation of cell-free extracts. The medium used was medium A as described in Section 1 of Chapter I. *R. erythropolis* D-1 was grown in 500 ml of medium A containing 50 mg/l DBT in a 2-l shaking flasks or 20 l of the same medium in a 30-l jar fermentor (Micros fermentor system, New Brunswick Scientific Co., Inc., Edison, U. S. A.). DBT was added to medium as the ethanol solution. For flasks, cultivation was done at 30°C for 2 days with reciprocal shaking, 100 strokes per min. For jar fermentor, it was done at 30 °C with continuous agitation and aeration, 700 rpm and 5 l per min, until DBT disappeared completely. Cells were harvested by continuous centrifugation at 10,000 x *g* and kept at -20°C until use. For the preparation of the cell-free extracts, 200 g (wet weight) of frozen cells were thawed and suspended in 400 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT), then disrupted with an ultrasonic oscillator (20 kHz, Sonifier 450, Branson Instruments, Danbury, U. S. A.). The cell debris was removed by centrifugation at 12,000 x *g* for 30 min. The supernatant was used as the cell-free extracts.

Purification of DBT oxygenase system. All purification steps were performed at 4°C. The buffer used throughout the purification contained 0.2 mM PMSF and 1 mM DTT.

(1) Step 1 $(NH_4)_2SO_4$ fractionation. The cell-free extracts was brought to 30% saturation with $(NH_4)_2SO_4$. After 2 h, the precipitate was removed by centrifugation (12,000 x *g*, 30 min) and the resultant supernatant was further saturated with $(NH_4)_2SO_4$ to 60% saturation. After 2 h, the precipitate was recovered by centrifugation (12,000 x *g*, 30 min), dissolved in a minimum volume of 50 mM Tris-HCl buffer (pH 8.0) and dialyzed overnight against the same buffer.

(2) Step 2 DEAE-Sepharose column chromatography. The dialyzed enzyme solution was put on a DEAE-Sepharose Fast Flow column (3.6 x 36 cm) which had been equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The column was washed well with 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM KCl, and the bound proteins were eluted with a linear gradient of KCl from 100 to 400 mM in the same buffer (gradient volume, 1,700 ml) at a flow rate 60 ml/h. No single fraction was active, but DBT degradation activity was observed when the fractions eluted at about 0.22 mM, designated as fraction A, were combined with the fractions eluted at about 0.27 mM, designated as fraction B. Fractions containing significant activity were pooled separately and precipitated by adding $(NH_4)_2SO_4$.

(3) Step 3 Phenyl-Toyopearl column chromatography. For the purification of component B, the resultant precipitant by adding $(NH_4)_2SO_4$ in step 2 was recovered by centrifugation (12,000 x *g*, 30 min), dissolved in a minimum volume of 50 mM Tris-HCl buffer (pH 8.0) containing 1 M $(NH_4)_2SO_4$. The enzyme solution was applied to a column of Phenyl-Toyopearl 650 M (2.5 x 5 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 1 M $(NH_4)_2SO_4$. The column was washed well with the same buffer, proteins were eluted with a linear gradient of $(NH_4)_2SO_4$ from 1 to 0 M in the same buffer (gradient volume, 170 ml) at a flow rate 30 ml/h. Fractions containing component B were combined and concentrated by ultrafiltration.

(4) Step 4 MonoQ chromatography. The MonoQ HR 10/10 column was equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl and loaded with the concentrated enzyme solution at step 3. Proteins were eluted with a linear gradient of NaCl from 200 to 450 mM in the same buffer (gradient volume, 80 ml) at a flow rate 2 ml/min controlled by FPLC system (Pharmacia, Uppsla, Sweden). Active fractions were concentrated by ultrafiltration.

Enzyme assays. The activity of DBT oxygenase system was assayed by measuring the amount of decreasing DBT at step 1 and 2 of purification and that of forming DBT sulfone after step 3 of purification. The standard reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 1 mM DBT, 3 mM NADH, 10 μM FMN, and a suitable amount of enzyme solution in a total volume of 0.5 ml. For the purification of component B, an excess of fractions A was used for the enzyme assay. One unit of enzyme activity was defined as the amount of protein that degraded 1 nmol of DBT or formed 1 nmol of DBT sulfone per min at 35°C.

NADH-linked reductase was assayed as the reduction of dichlorophenolineindophenol (DCPIP), cytochrome *c* and potassium hexacyanoferrate (ferricyanide). The reaction mixture contained 20 mM potassium phosphate buffer (pH 7.0), 0.4 mM NADH, and the artificial electron acceptor in a total volume of 1 ml. The concentrations of the artificial electron acceptors used were as follows: 0.1 mM DCPIP, 0.1 mM cytochrome *c* and 0.2 mM ferricyanide. The molar absorptivities for the reduction of DCPIP, cytochrome *c* and ferricyanide were taken to be 21,000 M^{-1} cm^{-1} at 600 nm [74], 21,000 M^{-1} cm^{-1} at 550 nm [75] and 1,020 M^{-1} cm^{-1} at 420 nm [76], respectively. One unit of reductase activities was expressed as the amount of enzyme that reduced 1 μmol of electron acceptor per min at 30°C.

Electrophoresis. Purification of component B was monitored by SDS-PAGE according to the method of Laemmli [77]. Slab gels (90 mm x 80 mm x 1 mm) with 12.5% polyacrylamide in the separating gel and 4% in the stacking gel were used for the electrophoresis, stained in 0.25% Coomasie Brilliant Blue G-250 dissolved in 50% methanol–10% acetic acid for 2 h and then destained in 30% methanol–10% acetic acid.

HPLC. For the determination of DBT and DBT sulfone, HPLC was performed as descried in Section 1 of Chapter I. For the determination of native molecular mass of proteins, a Superdex 200 HR 10/30 column was used at a flow rate 0.25 ml/min. The mobile phase was 50 mM Tris-HCl (pH 8.0) with 150 mM NaCl. This chromatography was controlled by FPLC system. The calibration protein used for the component B was thyrogloburin (Mr, 669,000), ferritin (Mr, 440,000), catalase (Mr, 232,000), aldolase (Mr, 158,000) and bovine serum albumin (Mr. 67,000).

Other analytical methods. N-Terminal amino acid sequences were determined by a PPSQ-10 protein sequencer (Shimadzu, Kyoto, Japan). Photometric assay was done with a UV-2200A spectrophotometer (Shimadzu, Kyoto, Japan). Protein concentrations were determined by the method of Bradford [56].

RESULTS AND DISCUSSION

Purification of DBT oxygenase system

DBT was converted to 2-HBP by the cell-free extracts. However, no activity was found in any single fractions at the stage of DEAE-Sepharose

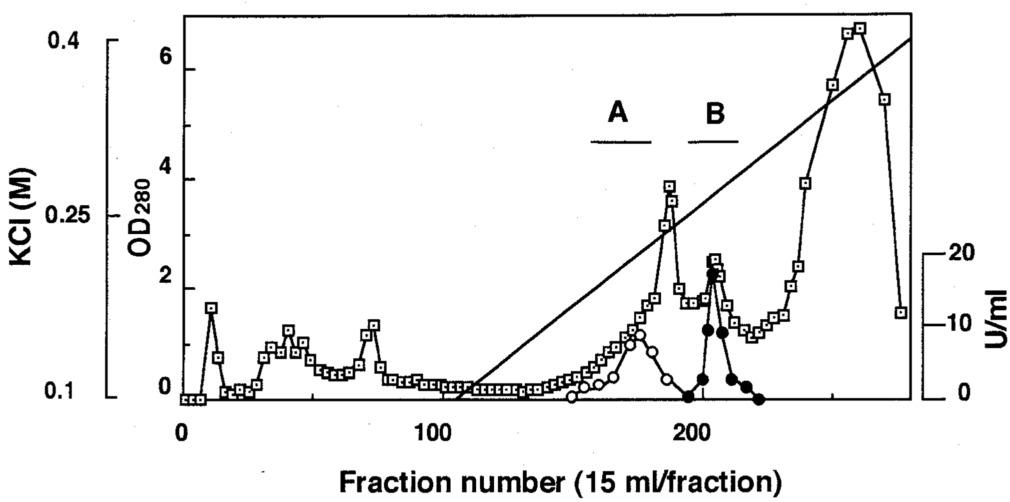


Fig. 1. Separation of Components of DBT Oxygenase System by DEAE-Sepharose Column Chromatography.

Proteins were eluted by a KCl gradient (——). Fraction A (○) was located by measuring DBT oxygenase activity of fractions in assay mixtures containing a crude component B (fraction no. 205). Fraction B (●) was located by testing the fractions for DBT oxygenase activity in the prresence of fraction A. The protein level was monitored as OD280 (□).

column chromatography as described in Materials and Methods. As shown in Fig. 1, DBT degrading activity could be detected when fraction A was combined with fraction B, and the product was DBT sulfone not 2-HBP. The activity catalyzing from DBT sulfone to 2-HBP was detected in other fractions. This column chromatography resulted in not only the separation of DBT oxygenase system into muticomponents enzyme but also the separation of the activity of degradation from DBT to 2-HBP into two or more enzymatic activities. Component B was first purified as the protein involved in DBT degradation, it was apparent homogeneous estimated by SDS-PAGE (Fig. 2). Table I summarizes the purification of component B.

Determination of molecular mass and N-terminal amino acids

The molecular mass of the component B of DBT oxygenase system was determined for native and denature proteins. Native component B had value of 250,000 and the denatured protein gave a molecular mass of 45,000.

Table I. Purification of Component B of DBT Oxygenase System

	Protein (mg)	Total activity (U)	Specific activity (U/mg)
Cell-free extracts	6715	7674	1.14
$(NH_4)_2SO_4$ fractionation	2407	4675	1.94
DEAE-Sepharose	236	1285	5.44
Phenyl-Toyopearl	46	513	11.2
MonoQ	16	187	11.6

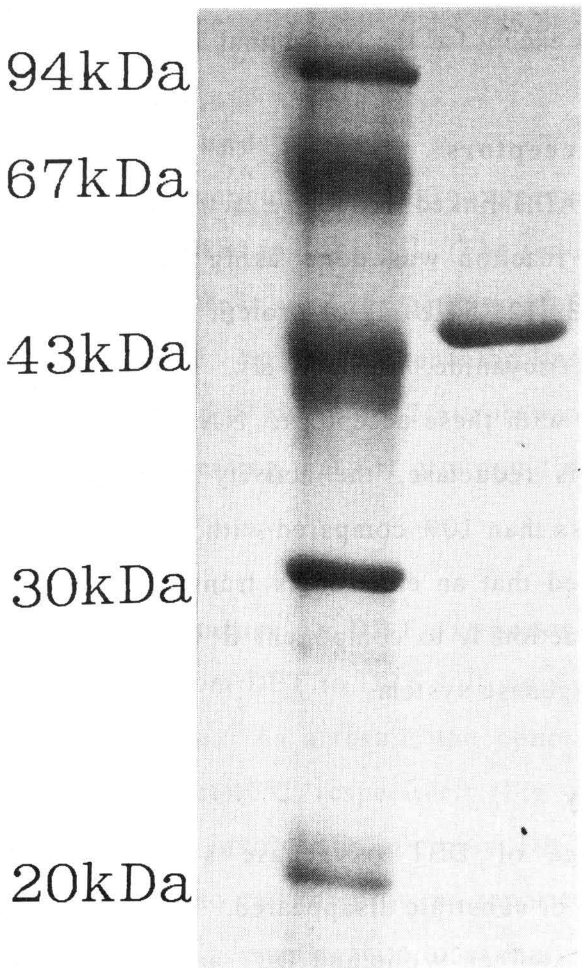


Fig. 2 SDS-PAGE of Component B.

Therefore, component B was presumed to be identical hexamer. Some muticomponent oxygenase systems [78-81] have been reported, subunit molecular mass of component B seemed to be similar with that of other oxygenase systems. However, hexamer structure as oxygenase has not yet been demonstrated.

The amino acids sequence of the amino terminus of component B was Thr-Leu-Ser-Pro-Glu-Lys-Gln-His-Val-Arg-Pro-Arg-Asp-Ala-Ala-Asp-Asn-Asp-Pro-Val, this sequence entirely agreed with the predicted sequence from *soxC* or *dbzC* genes [59, 82] except for the N-terminal amino acid, methionine.

Artificial electron acceptors

Fraction A had NADH-linked reductase activity with artificial electron acceptors. When the reaction was done using fraction A as the enzyme, specific activities of 63, 12, 54 U/mg of protein were observed with DCPIP, cytochrome *c*, and ferricyanide, respectively. Component B showed no oxidoreductase activity with these acceptors. NADPH was not effective as an electron donor for this reductase, the activity for NADPH in the DCPIP reductase assay was less than 10% compared with that for NADH. From these results, it was proposed that an electron is transferred from NADH via the protein contained in fraction A to component B which acts as the oxygenase component of DBT oxygenase system.

Substrate specificity

The substrate range of DBT oxygenase system was investigated by measuring the amounts of substrate disappeared. This enzyme system acts not only DBT but also thioxanthen-9-one and DBT analogues, 4,6-dimethyl DBT, 2,8-dimethyl DBT and 3,4-benzo DBT (Table II).

Table II. Substrate Specificity of DBT Oxygenase System
The enzyme reaction was done using 90 μg of fraction A and 120 μg of component B. Each compound was added at 1 mM.

Componuds	Relative activity (%)
DBT	100
3,4-Benzo DBT	6.3
2,8-Dimethyl DBT	129.2
4,6-Dimethyl DBT	52.1
Biphenyl	0
Carbazole	0
Dibenzofurane	0
Fluorene	0
Thioxanthen-9-one	45.2

Effects of several compounds

The effects of various compounds on DBT oxygenase system and NADH-linked reductase are summarized in Table III. The reductase was inhibited by some metal ions, Cu^{2+} and Mn^{2+}, and the activity of DBT oxygenase system was significantly inhibited by 1,10-phenanthroline, 2,2'-bipyridyl, *p*-chloromercuribenzoic acid, 5,5'-dithiobis(2-nitrobenzoic acid), 8-qunolinol, Mn^{2+}, Cu^{2+}, and Zn^{2+}, suggesting that SH group and metal might involved in the oxygenase activity.

Effects of pH and temperature on DBT oxygenase activity

The catalyzing activity from DBT to DBT sulfone was examined in various range of pH and temperature. As a result, the optimal pH was 8, and the optimal temperature was about 40°C, respectively (Fig. 3).

In this work, the protein involved in microbial DBT degradation has been first purified. I would like to call the proteins reported here DBT oxygenase system, their structure might be similar with other muticomponent oxygenases. This enzyme system catalyzing the reaction from DBT to DBT sulfone was the initial enzymes for DBT degradation and could be regarded as the proteins

Table III. Effects of Various Compounds on the Enzyme Activities

For reductase assay the enzyme reaction was done using 180 μg of fraction A and 0.4 mM NADH. For oxygenase assay the reaction mixture contained 90 μg of fraction A and 120 μg of component B.

Compounds	Concentration (mM)	Relative activity (%)	
		reductase	oxygenase
None		100	100
2, 2'-Bipyridyl	1.0	77.9	2.4
	0.1	N.D	68.0
PCMB[a)]	1.0	145.8	1.3
	0.1	N.D	16.3
DTNB[b)]	0.5	73.8	23.8
	0.05	N.D	30.0
EDTA[c)]	1.0	N.D	100.6
N-Ethylmaleimide	1.0	N.D	103.0
Iodoacetate	1.0	N.D	99.1
1, 10-Phenanthroline	1.0	90.3	0
	0.1	N.D	13.9
Potassium fluoride	1.0	N.D	97.5
8-Quinolinol	0.1	74.3	50.2
	0.01	N.D	100.2
$CaCl_2$	1.0	N.D	80.3
$CoSO_4$	1.0	100.3	65.2
	0.1	N.D	100.7
$CuSO_4$	1.0	0	0
	0.1	N.D	15.0
$FeSO_4$	1.0	N.D	108.1
$MnSO_4$	1.0	51.3	36.3
	0.1	N.D	36.0
$ZnSO_4$	1.0	78.2	5.7
	0.1	N.D	20.4

a) *p*-Chloromercuribenzoic acid
b) 5, 5'-Dithiobis(2-nitrobenzoic acid)
c) Ethylenediaminetetraacetic acid
N.D: not determined

activating sulfur atom of DBT molecule in order to cleave C-S bond of DBT molecule at the subsequent reaction.

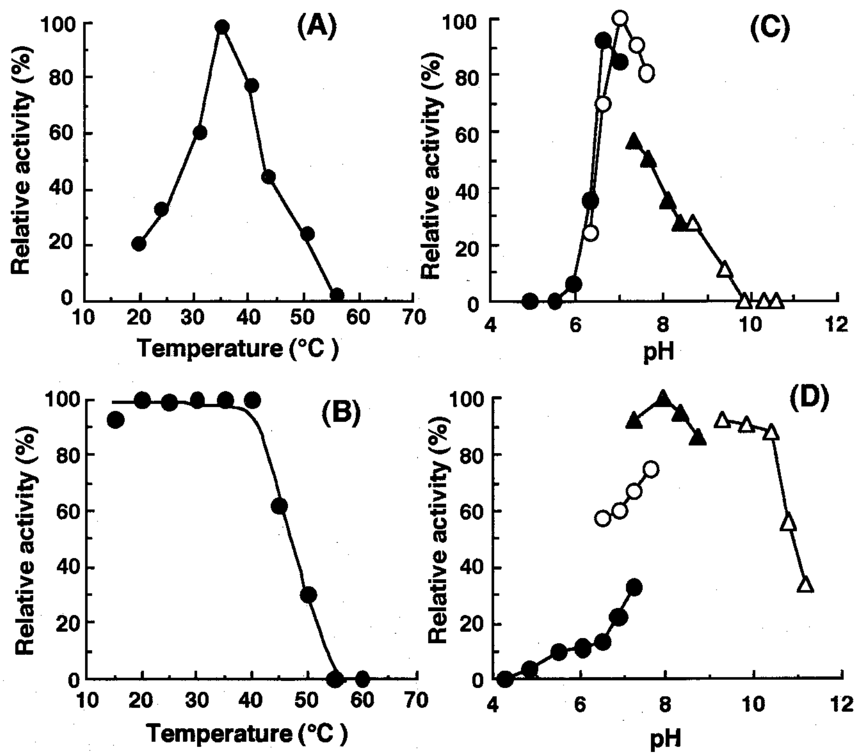


Fig. 3. Effects of Temperature and pH on the Activity (A, C) and the Stability (B, D) of DBT Oxygenase System.

To examine the enzyme activity under different temperature, the reaction was done under the standard conditions using 90 μg of fraction A and 120 μg of component B except the reaction temperature. To examine the enzyme stability under different temperature, 90 μg of fraction A and 120 μg of component B were incubated in the indicated temperature for 30 min. To examine the enzyme activity under different pH, the reaction mixture was incubated under the standard conditions using 90 μg of fraction A and 120 μg of component B except the reaction buffer. To examine the enzyme stability under different pH, 90 μg of fraction A and 120 μg of component B were incubated in the indicated buffers for 30 min. After dialysis against 100 mM potassium phosphate buffer (pH 7.0), the enzyme activity was measured under the standard reaction conditions. Buffers: ●, citrate-phosphate buffer; ○, potassium-phosphate buffer; ▲, Tris-HCl buffer; △, glycine-NaOH buffer.

SUMMARY

The enzyme proteins, DBT oxygenase system, involved in the conversion of DBT to DBT sulfone were separated into the two fractions (fraction A and fraction B) at the purification stage by DEAE-Sepharose column chromatography. One enzyme protein, designated as component B, was purified to homogeneity from *R. erythropolis* D-1. The molecular mass of the component B was 250 kDa and it consisted of identical six subunits with molecular weight of 45 kDa. Another enzyme protein(s) contained in fraction A has NADH-linked reductase activity with artificial electron acceptors. DBT oxygenase system exhibited a narrow substrate specificity, it acted on some DBT derivatives but not on carbazole and fluorene. The activity of DBT oxygenase system was inhibited by 1,10-phenanthroline, 2,2'-bipyridyl, *p*-chloromercuribenzoic acid, 5,5'-dithiobis(2-nitrobenzoic acid), 8-qunolinol, Mn^{2+}, Cu^{2+}, and Zn^{2+}, suggesting that thiol group and metal might related to the enzyme activity. The optimal temperature of this enzyme system was 40°C and the optimal pH was about 8.0.

CHAPTER III
Microbial Sulfur Insertion for Biotin

Section 1. Enzymatic Conversion of Dethiobiotin to Biotin in Cell-free Extracts of *Bacillus sphaericus*

The enzymes involved in microbial biotin biosynthesis, from pimelic acid to dethiobiotin (DTB), have been already investigated in detail [32, 33, 83-87]. However, the enzyme(s) for the last step, the conversion of DTB to biotin, is still unknown. This step has received great attention from the standpoint of organic chemistry as well as enzymology because the sulfur atom is introduced at non-activated position between the methylene group at C-6 and the methyl group at C-9 of the dethiobiotin skeleton. The mechanisms of the last step in biotin biosynthesis and the origin of sulfur in biotin have been discussed by some investigators, but no definitive solution was proposed [88-93]. Recently, putative intermediates thiols were prepared chemically [94, 95] and it was found that the primary thiol at C-9 was converted into biotin by *Escherichia coli* and *Bacillus sphaericus* [96]. Moreover, in plant cells cultures, an intermediate which is very likely the C-9 thiol was isolated and converted into biotin [97].

It has been revealed that the genes involved in biotin biosynthesis existed as the biotin operon [98, 99]. Recombinant strains of *B. sphaericus*, *E. coli* and *Serratia marcescens* have been constructed by cloning the genes to overproduce biotin [100-104]. The enzyme encoded by *bioB* is considered to catalyze the conversion of DTB to biotin, and the *bioB* transformants of *B. sphaericus* and *E. coli* were also constructed to elucidate the last step of biotin biosynthesis [105, 106]. The conversion of DTB to biotin has been reported in cell-free extracts of a *bioB* transformant of *E. coli* [106], and in resting cells [107], protoplasts [107] and cell-free extracts [108] of a *bioB* transformant of *B. sphaericus*.

This section describes the enzymatic conversion of DTB to biotin by cell-free extracts of a *bioB* transformant of *B. sphaericus* with a completely defined reaction mixture, and demonstrates possible cofactors required for the enzymatic reaction.

MATERIAL AND METHODS

Microorganisms and culture conditions. *B. sphaericus* BT(250)C (*bioR*$^-$, actithiazic acid^r, 5-(2-thienyl)-valeric acid^r, 1-(2'-thenoyl)-3,3,3-trifluoroacetone^r), derived from *B. sphaericus* IFO3525 [31], was transformed with pBHB5022 [100] which contains a *bioB* fragment. The transformant BT(250)C[pBHB5022] was grown for 24 h at 30°C in GP medium [25] with a slight modification (20 g of glycerol, 30 g of peptone (Wako), 5 g of vitamin-free Casamino acids (Difco), 1 g of K_2HPO_4, 0.5 g of KCl, 0.5 g of $MgSO_4{\bullet}7H_2O$, 0.01 g of $MnSO_4{\bullet}4\text{-}6\ H_2O$, and 20 mg of thiamin•HCl in 1 liter, pH 7.0). Cultivation was done at 30°C for 40 h in 2-liter flasks containing 800 ml of medium with reciprocal shaking (100 strokes/min). The cells were harvested from the culture broth by centrifugation at 10,000 x *g* for 15 min, washed once with 0.85% NaCl, and kept at -20°C until use.

Preparation of cell-free extracts. All operations were done below 15°C. Twenty-five grams (wet weight) of frozen cells were suspended in 50 ml of 60 mM Tris-HCl buffer (pH 8.0) and treated with an ultrasonic oscillator (20 kHz). The cell debris were removed by centrifugation at 12,000 x *g* for 30 min. The supernatant solutions were used as the cell-free extracts.

Preparation of dialyzed cell-free extracts. The cell-free extracts were brought to 90% saturation with ammonium sulfate. After 1 h the resulting precipitate was collected by centrifugation at 12,000 x *g* for 30 min, dissolved in 60 mM Tris-HCl buffer (pH 8.0), and dialyzed overnight against three changes of a total volume of 6 liters of the same buffer. The resultant solution was used as the dialyzed cell-free extracts.

Enzyme assay and protein determination. The enzyme assay of biotin synthase was done by measuring the amount of biotin formed from DTB. The standard reaction mixture contained 40 mM Tris-HCl buffer (pH 8.0), enzyme, 0.2 mM DL-DTB, and various other compounds in a final volume of 500 μl, and the reaction mixture was incubated at 37°C for 60 min. Biotin was measured by a microbiological method (paper disk plate method) with *Lactobacillus plantarum* [109]. Protein was measured by the method of Bradford [56] with bovine serum albumin as a standard. Activity was expressed as the amount of biotin that was formed by 1 mg of protein per h.

Chemicals. *S*-Adenosyl-L-methionine (*p*-toluenesulfonate salt) was purchased from Sigma Chemical Co., St. Louis, U.S.A. and was kindly supplied by Nippon Zeon Co., Kawasaki, Japan. Methylthioadenosine was also a gift from Nippon Zeon Co. All other chemicals were of reagent grade.

RESULTS

Effects of sulfur compounds on the enzymatic synthesis of biotin from DTB in the cell-free extracts

Since only a weak biotin synthase activity was detected when the cell-free extracts of *B. sphaericus* were incubated with DTB alone, various sulfur-containing compounds were examined to find whether they could act as sulfur donors in the biotin synthase reaction. Among the sulfur compounds tested, only *S*-adenosyl-L-methionine (AdoMet) had a significant effect on biotin synthesis from DTB (Table I). Methionine and cysteine were inert.

Table I. Effects of Sulfur Compounds

The reaction mixture contained 40 mM Tris-HCl buffer (pH 8.0), 0.2 mM DL-DTB, the cell-free extracts (29.7 mg/ml of protein), and the indicated sulfur compound at 1 mM.

Sulfur compound	Biotin formed (nmol/mg•h)
None	0.05
L-Methionine	0.09
D-Methionine	0.08
L-Cysteine	0.08
D-Cysteine	0.07
DL-Homocysteine	0.06
S-Adenosyl-L-methionine	1.30
S-Adenosyl-L-homocysteine	0.06
Methylthioadenosine	0.07
S-Sulfocysteine	0.07
L-Cysteine sulifinic acid	0.07
L-Cysteinic acid	0.08
Cysteamine	0.07
Taurine	0.07
Glutathione	0.07

Effects of protein and substrate concentrations

Some properties of biotin synthase in the cell-free extracts were investigated by examining the effects of protein, DTB, and AdoMet concentrations on the activity. First, the enzymatic reaction was done in the presence of 1 mM AdoMet and the cell-free extracts containing various amounts of protein. Though biotin synthase activity was not detectable at less than 10 mg protein/ml, it was clearly observed at more than 15 mg protein/ml (Fig. 1A). The activity markedly increased as protein concentrations were increased between 15 and 20 mg/ml. Furthermore, as shown in Figs. 1B and 1C, the activity was enhanced by increasing DTB and AdoMet concentrations up to 100 μM and 500 μM, respectively, in the reaction conditions.

Biotin was formed linearly with reaction time, reaching a plateau after 20 min in the presence of 1 mM AdoMet (Fig. 2). Assuming that the end of biotin production could be due to the consumption of some cofactors, I dialyzed the cell-free extracts thoroughly as described in Materials and Methods and examined the requirements for cofactors and metal ions for biotin synthase of this strain in the following experiments.

Requirements for biotin synthase activity

Total protein in the cell-free extracts was once precipitated by adding ammonium sulfate to 90% saturation and then dialyzed thoroughly to exclude low molecular weight compounds. Little activity was detected in the dialyzed cell-free extracts even in the presence of 1 mM AdoMet. Therefore, the effects of various well-known cofactors on biotin synthase activity were investigated. NAD^+, NADH, $NADP^+$, NADPH, and FAD restored the activity as shown in Table II.

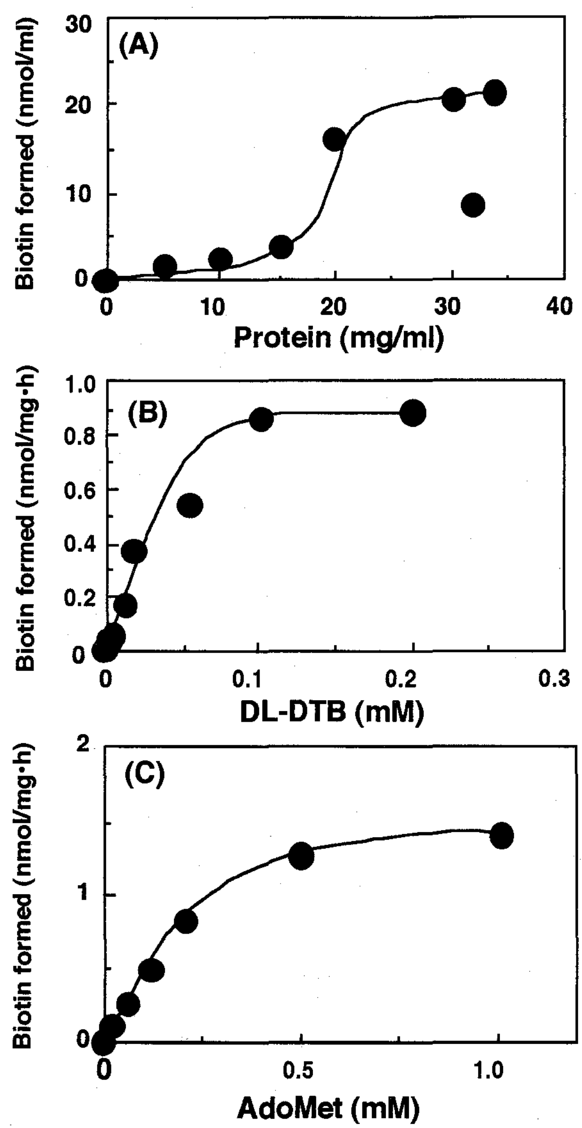


Fig. 1. Effects of Protein, DTB, and AdoMet Concentrations on the Activity.
(A) The reaction mixture contained 40 mM Tris-HCl buffer (pH 8.0), 0.2 mM DL-DTB, 1 mM AdoMet, and the cell-free extracts of the indicated protein concentrations. The amount of biotin formed was measured after 1 h of incubation. (B) The reaction mixture contained 40 mM Tris-HCl buffer (pH 8.0), 1 mM AdoMet, the cell-free extracts (23.3 mg/ml of protein), and different concentrations of DL-DTB. (C) The reaction mixture contained 40 mM Tris-HCl buffer (pH 8.0), 0.2 mM DL-DTB, the cell-free extracts (23.3 mg/ml of protein), and different concentrations of AdoMet.

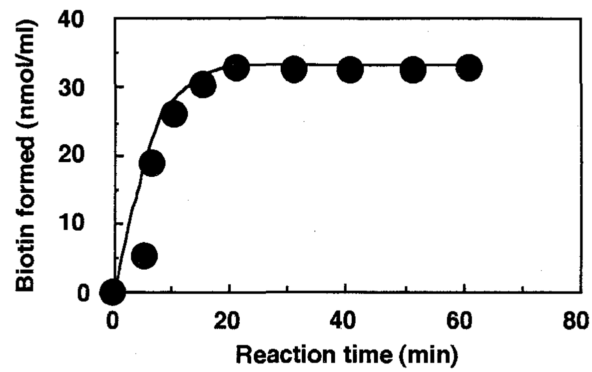


Fig. 2. Course of Biotin Synthesis from DTB by Cell-free Extracts.

The reaction mixture contained 40 mM Tris-HCl buffer (pH 8.0), 0.2 mM DL-DTB, 1mM AdoMet, and the cell-free extracts (33.1 mg /ml of protein).

Table II. Effects of Cofactors

The reaction mixture contained 40 mM Tris-HCl buffer (pH 8.0), the dialyzed cell-free extracts (14 mg/ml of protein), 0.2 mM DL-DTB, 1 mM AdoMet, and the indicated compound at 1 mM.

Compound	Biotin formed (nmol/mg•h)
None	0.02
NAD	0.16
NADH$^+$	0.30
NADP	0.19
NADPH$^+$	0.37
FAD	0.20
FMN	0.01
Pyrroloquinoline quinone	0.02
Cytochrome *c*	0.02
Phenazine ethosulfate	0.01
Pyridoxal 5 -phosphate	0.04
CoA (reduced form)	0.04
Fructose-1,6-diphosphate	0.02

Effects of coenzymes concentrations

The concentration effects of four pyridine nucleotide coenzymes and FAD were examined. The maximum activity was reached at a coenzyme concentration of 1 mM (Fig. 3). Reduced forms of pyridine nucleotide coenzymes, NADH and NADPH, were more effective than the oxidized forms. Furthermore, the simultaneous addition of both 1 mM NAD(P)H and 1 mM FAD stimulated biotin synthesis about 1.5 times compared with the addition of NAD(P)H alone and FAD alone, respectively (data not shown).

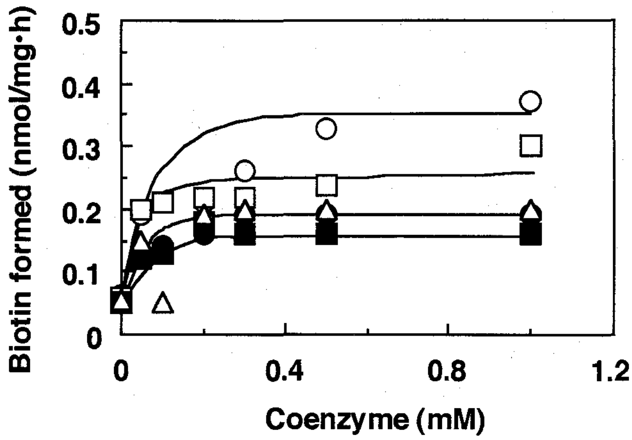


Fig. 3. Effects of Coenzymes Concentrations on the Activity.

The reaction mixture contained 40 mM Tris-HCl buffer (pH 8.0), 0.2 mM DL-DTB, 1 mM AdoMet, the dialyzed cell-free extracts (14.0 mg/ml of protein), and different concentrations of NADH (□), NAD (■), NADPH (○), NADP (●), and FAD (△).

Effects of metal ions

The effects of metal ions were examined in the presence of 1 mM AdoMet, 1 mM NADPH, and 1 mM FAD. Among the metal ions tested, Fe^{2+}, Mn^{2+}, and Ca^{2+} were effective (Table III), but a concentration of Fe^{2+} higher than 1 mM was inhibitory (data not shown).

Table III. Effects of Metal Ions

The reaction mixture contained 40 mM Tris-HCl buffer (pH 8.0), the dialyzed cell-free extracts (19 mg/ml of protein), 0.2 mM DL-DTB, 1 mM AdoMet, 1mM NADPH, 1 mM FAD, and the indicated metal ions at 1 mM.

Metal salt	Biotin formed (nmol/mg•h)
None	0.20
$Al_2(SO_4)_3$	0.16
$BaCl_2$	0.30
$CaCl_2$	0.46
$CdCl_2$	0.03
$CoSO_4$	0.04
$CuCl_2$	0.03
$FeSO_4$	0.45
$FeCl_3$	0.38
$MgSO_4$	0.28
$MnCl_2$	0.53
Na_2MoO_4	0.05
$NiCl_2$	0.05
$(CH_3COO)_2Pb$	0.03
$ZnCl_2$	0.06
KCl	0.50
LiCl	0.35
NaCl	0.19

DISCUSSION

B. sphaericus has been reported to be a potent biotin producer [127]. The final step of biotin biosynthesis, the conversion of DTB to biotin, has recently been demonstrated in cell-free extracts of this strain [108]. However, the reaction mixture contained yeast extract. In this section, I have presented biotin synthesis from DTB in a simple system with cell-free extracts of *B. sphaericus* , without yeast extract. The relation between protein concentration and the activity was observed to be unusual (Fig. 1A) as previously reported by Fujisawa *et. al.* [108]. The reason for this still remains to be solved. This could be due to the requirement of at least another protein or a multienzymatic complex. It is considered that the requirement for the extraordinary high protein concentrations in the detection of the activity made it very difficult to find biotin synthase activities in cell-free extracts.

In *E. coli* cell-free extracts, the conversion of DTB to biotin has already been reported [106]. However, there are some differences between *B. sphaericus* and *E. coli* in cofactor requirements. First, I have noticed that among the sulfur compounds tested, only AdoMet was active for biotin synthase in *B. sphaericus*, but in *E. coli*, L-methionine as well as AdoMet was active. This may suggest the presence of an AdoMet synthase that might catalyze the synthesis of AdoMet from L-methionine in *E. coli*. It was previously shown that the sulfur of L-methionine was incorporated into the biotin molecule with resting cells of other microorganisms, *Saccharomyces cerevisiae* [110] and *Rhodotorula glutinis* [111]. In addition, fructose 1,6-diphosphate and KCl seemed to be possible cofactors of biotin synthase in *E. coli*. I observed the same result with *B. sphaericus* cell-free extracts for KCl, but fructose 1,6-bisphosphate was not effective. On the other hand, FAD was found to have a significant effect on the activity. This is the first result concerning the participation of FAD in biotin synthesis. Fe^{2+} also had effects on the activity just like in the *E. coli* enzyme. I can conclude that biotin synthase in *B. sphaericus* probably used at least NAD(P)H and FAD as cofactors and Fe^{2+} might be involved in the enzyme reaction.

SUMMARY

The activity of biotin synthase, responsible for biotin synthesis from dethiobiotin, was demonstrated in a completely defined reaction mixture with cell-free extracts of a *B. sphaericus bioB* transformant. Among the sulfur compounds tested, only *S*-adenosyl-L-methionine was active, while L-methionine and L-cysteine had no significant effect. Protein concentrations

higher than 15 mg/ml in the reaction mixture were needed to detect biotin synthase activity. When dialyzed cell-free extracts were used for the reaction, NADH, NADPH, or FAD among the well-known cofactors tested enhanced the activity, and Fe^{2+}, Mn^{2+} and Ca^{2+} among the metal ions tested also had some effects.

Section 2. Involvement of Cysteine on Biotin Synthase of *Bacillus sphaericus*

The last step of biotin biosynthesis, namely, the introduction of sulfur into dethiobiotin (Fig. 1) has long raised a very puzzling problem in spite of intensive studies [88, 89, 93, 96]. This has been due to the fact that all the experiments were performed with whole cells, no active cell-free system having been obtained. Looking for the origin of sulfur, many possible sulfur donors were tested and it was found that only *S*-adenosyl methionine (AdoMet) efficiently increased the activity as described in Section 1 of this Chapter. However, it was thought that from a mechanistic point of view, it was difficult to consider AdoMet as a sulfur donor in this reaction and I present here experiments which establish that indeed, AdoMet is playing another role.

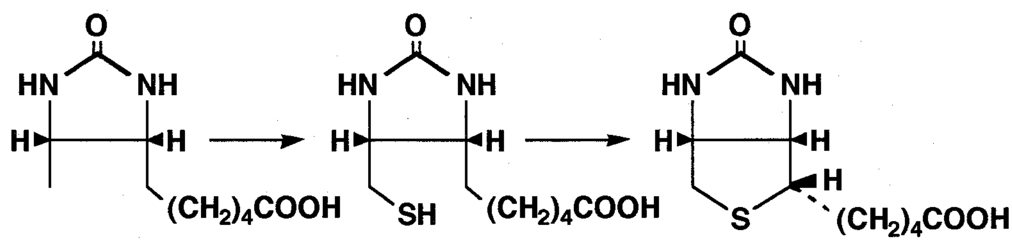


Fig. 1. Introduction of Sulfur into Dethiobiotin

MATERIALS AND METHODS

All chemicals were of the highest purity available. Cysteine, methionine, AdoMet and (±)-dethiobiotin were purchased from Sigma, [^{35}S] methionine and [^{35}S] cysteine from Radiochemical Center Ltd. Amersham. [^{34}S]

Mercaptodethiobiotin was synhthesized according to the previously reported method [96].

B. sphaericus having *bioB* fragment was aerobically cultured at 30°C for 40 h in GP medium as described in Section 1 of this Chapter. Preparation of cell-free extracts, determinations of biotin formed and protein were also performed as described in Section 1 of this Chapter.

In the reaction using [^{34}S] mercaptodethiobiotin 1, the reaction mixture contained 60 μM [^{34}S] mercaptodethiobiotin, 120 μM dithiothreitol, 40 mM Tris-HCl buffer (pH 8.0), 1 mM AdoMet and cell-free extracts (15 mg protein/ml) in a final volume of 500 μl. The reaction mixture was incubated at 37°C for 180 min. Reaction mixtures were collected from 190 experiments. After centrifugation, the supernatnt was lyophilized. The protein were precipitated with ethanol. After evaporation, H_2O (100 ml) was added, the pH of the solution adjusted to 3-4 and the solution stirred with 6 g Norit overnight. After filtration, Norit was eluted with $NH_4OH/H_2O/C_2H_5OH$ (1/10/10). The eluate was concentrated to 20 ml under vacuum. The solution was chromatographed on a DOWEX 1 x 2.200 column, eluted with a linear gradient of formic acid (0.05 to 0.1 N). Biotin in the fractions was detected by the microbiological test using *Lactobacillus plantarum*. The fractions containing biotin were lyophilized. After esterification and purification on a silica-gel column ($CHCl_3/CH_3OH$, 9/1) followed by HPLC (RP8 column 10 x 0.32, eluent CH_3OH/H_2O, 4/6, 0.8 ml/min), about 5 μg of biotin were obtained and studied by mass spectrometry. The determination of the [^{32}S]/[^{34}S] ratio was calcuated on the parents peaks obtained from the daughter common ion: m/z 55 [96].

In the reaction using [^{35}S] cysteine, the reaction mixture contained 200 μM ($\pm$)-DTB, 160 μM cysteine (S.A. :34 mCi/mmol), 160 μM dithiothreitol, 400 μM ATP, 400 μM $MgCl_2$, 1 mM AdoMet, 40 mM Tris-HCl buffer (pH 8.0) and cell-free extract (30 mg protein/ml) in a final volume of 500 μl. The reaction mixture was incubated at 37°C for 60 min. Reaction mixtures were collected from 34 experiments and treated as above. The fractions corresponding to biotin after the Dowex column were lyophilized, taken in 1 ml water and checked by TLC ($CHCl_3/CH_3OH/CH_3COOH$: 9/1/0.5) and TLC Linear Analyser LB 2852 (Berthold). The amount of biotin quantified after the Dowex column with *L. plantarum* as 130 $\pm$ 30 nmols and radioactivity corresponding to biotin on TLC Linear Analyser was 48% of total radioactivity, corresponding to a specific activity of 1.5 $\pm$ 0.4 mCi/mmol. The crude biotin was diluted with 50 mg of cold biotin and recrystallized three times in water. The four samples had the same specific activity, respectively, 956, 920, 930, 940 $\pm$ 20 nCi/mmol.

[^{35}S] AdoMet was prepared with [^{35}S] methionine (S. A. =228 mCi/mmol) according to the method proposed by P. Frey, which is a modification of a procedure of Markham *et al.* [112]. After dilution with cold AdoMet (6.4 mg), [^{35}S] AdoMet with S. A. of 24.3 mCi/mmol was used. The reaction mixture contained 1 mM AdoMet, 200 μM ($\pm$)-DTB, 40 mM Tris-HCl buffer (pH 8.0) and cell-free extract (25 mg protein/ml) in a final volume of 500 μl. The mixture was incubated at 37°C for 60 min and the reaction mixtures were collected from 16 experiments. Treatment was the same as for [^{35}S] cysteine. After the Dowex column, the amount of biotin quantified with *L. plantarum* was 16 $\pm$ 2 nmoles and with TLC Linear Analyser, no radioactivity was detected in the fractions corresponding to biotin.

RESULTS

It has been shown that mercaptodethiobiotin 1 (Fig. 1, DTBSH), labelled with ^{35}S or ^{34}S was converted into biotin by resting cells of *B. sphaericus*, with conversion of about 75% of the label. It has been thus postulated that 1 was very likely an intermediate between DTB and biotin. A compound very likely identical to 1 has been isolated from higher plants cells [97].

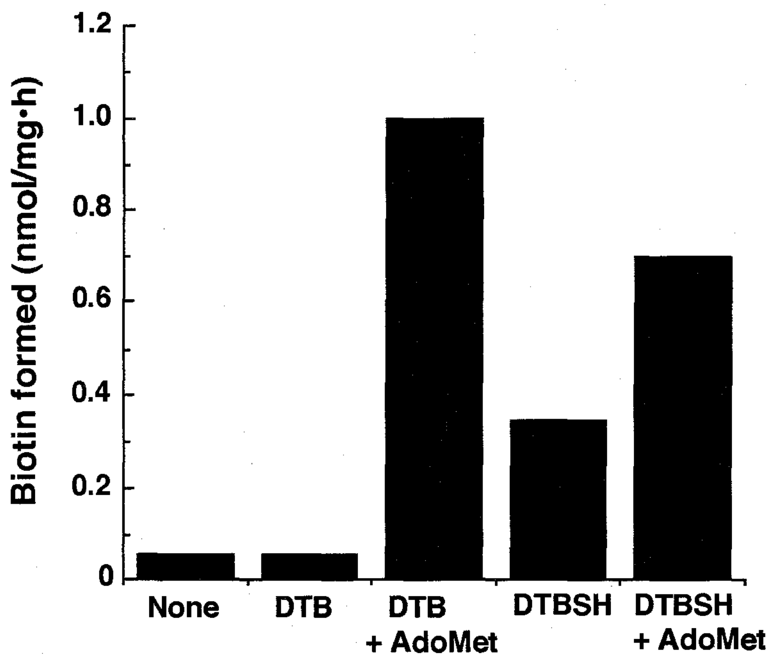


Fig. 2. Influence of AdoMet on the Transformation of DTB and Mercaptodethiobiotin into Biotin by Cell-Free Extracts of a *bioB* Transformant of *B. sphaericus*.

The reaction mixture contained the cell-free extract (36 mg/ml of protein), 40 mM Tris-HCl buffer (pH 8.0), 1 mM AdoMet and either 0.2 mM (+)-DTB or 0.1 mM mercaptodethiobiotin in a final volume 500 μl and the reaction mixture was incubated at 37°C for 60 min.

I have now examined the conversion of 1 with a cell-free system and I have found that AdoMet improved the activity, with both thiol and dethiobiotin (Fig. 2). However, using [^{34}S] 1 and unlabelled AdoMet, I obtained biotin which contained, according to the mass spectrum, about 75% of ^{34}S. This demonstrated that AdoMet is not in that case the sulfur donor. It is interesting to point out that the loss of ^{34}S is of the same order of magnitude with the cell-free and resting cells systems.

To confirm that AdoMet is not the sulfur donor, I prepared [^{35}S] AdoMet from [^{35}S] methionine, using AdoMet synthetase. A sample of [^{35}S] AdoMet with a specific activity of 24.3 mCi/mmol was used as cofactor for the conversion of DTB. The biotin which was isolated as completely devoid of radioactivity. The same experiment was run with cold AdoMet and [^{35}S] cysteine (S. A.: 34 mCi/mmol). In that case, radioactive biotin was obtained (S. A.: 1.5 $\pm$ 0.4 mCi/mmol). This result has of course no quantitative significance since it was obtained with a crude cell-free system.

DISCUSSION

I have cleary proved that AdoMet, which is absolutely necessary for the tranformation of DTB into biotin, does not act as the sulfur donor. Therefore, I would like to propose one working hypothesis that biotin synthase belongs to a family of enzymes which use AdoMet as a source of deoxyadenosyl radical with the possible relay of a protein radical, such as anaerobic ribonucleotide reductase [113-115], pyruvate-formate lyase [116, 117] or lysine 2,3-aminomutase [118]. The common feature of all these reactions is the homolytic cleavage of a C-H bond. All these enzymes contain a metallic center, either a [Fe-S] cluster or metallic ions in their active site and require NADPH and a flavoprotein. The requirement for NADPH and FAD has also been demonstrated in Section 1 of this Chapter. The occurence of a [2Fe-2S] cluster has also been postulated for the biotin synthase of *E. coli* [119].

SUMMARY

In a cell-free system of a *bioB* transformant of *B. sphaericus*, the introduction of sulfur into dethiobiotin, the last step of biotin biosynthesis, was investigated using sulfur labelled compounds. It was shown, through with [^{35}S] *S*-adenosylmethionine and [^{35}S] cysteine, that the sulfur donor is not *S*-adenosylmethionine but probably cysteine or its derivative. This finding together with the fact that NADPH and FAD are required for activity leads to postulate some analogy between the biotin synthase and other systems which use *S*-adenosylmethionine as a souurce of deoxyadenosyl radical.

Section 3. Stimulatory Factors for Enzymatic Biotin Synthesis from Dethiobiotin in Cell-free Extracts of *Escherichia coli*

Recently, the *bioB* gene product has been purified to nearly homogeneity from the *bioB* transformant of *E. coli.* It was reported to contain a [2Fe-2S] cluster and was active in the presence of crude extract of *E. coli* [120]. Ifuku *et al.* has demonstrated that flavodoxin is required for the conversion of dethiobiotin to biotin by the *bioB* product in *E. coli* [121]. In *B. sphaericus*, it has been demonstrated that AdoMet, NADPH and FAD had some influences on the enzymatic conversion as shown in Section 1 of this Chapter, and cysteine or its derivatives might be the sulfur donor through the experiments with [^{35}S] cysteine as shown in Section 2 of this Chapter. In *E. coli*, the effects of AdoMet and NADPH were evidenced [106], however I know of no report concerning the involvement of cysteine. This section presents an essential sulfur compound and possible cofactors for the enzymatic conversion of DTB to biotin by cell-free extracts of a *bioB* transformant of *E. coli* and also demonstrates that cysteine enhanced this activity.

MATERIALS AND METHODS

E. coli TK101 (α-dehydrobiotin^{r}) harboring the high expression vector of *bioB* was obtained from Nippon Soda Co. Ltd. The microorganism was grown for 24 h at 30°C in GP medium (20 g of glycerol, 30 g of Polypepton (Wako Chemicals, Osaka, Japan), 5 g of vitamin-free Casamino acids (Difco, Detroit, U. S. A), 1 g of K_2HPO_4, 0.5 g of KCl, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of $MnSO_4 \cdot 5H_2O$, 0.1 g of thiamin•HCl in 1 liter, pH 7.0 supplemented with 50 mg/l ampicillin and 0.2 mM isopropylthiogalactoside.

Cultivation was done for 24 h in 2-liter flasks containing 500 ml of medium with reciprocal shaking (100 strokes/min). The cells were harvested by continuous flow centrifugation (10,000 rpm), then washed with 0.85% NaCl, and stored at -20°C until use. Frozen cells (25 g, wet weight) were suspended in 50 ml of 60 mM Tris-HCl buffer (pH 8.0) and disrupted with an ultrasonic oscillator (Branson Sonifier 450). The cell debris was removed by centrifugation at 12,000 x *g* for 30 min. The supernatant solution is referred to as the cell-free extracts. Solid ammonium sulfate was added to the cell-free extracts to 0.90 saturation. The resulting precipitate was collected by centrifugation at 12,000 x *g* for 30 min dissolved in 60 mM Tris-HCl buffer (pH 8.0), and dialyzed against three changes of a total volume of 6 liters of the same buffer. The resultant solution is referred to as the dialyzed cell-free extracts. The enzyme assay was performed in a standard reaction mixture containing 20 μmol of Tris-HCl buffer (pH 8.0), 100 nmol of DL-DTB, and enzyme in a total volume of 500 μl. The reaction was carried out for 1 h at 37°C and the amount of biotin formed was microbiologically determined as described in Section 1 of this Chapter. The protein concentration was measured by the method of Bradford [56] with bovine serum albumin as a standard. Enzyme activity was expressed as the amount of biotin that was formed by 1 mg protein per h.

RESULTS AND DISCUSSION

No biotin forming activity was found when only DTB was incubated with the cell-free extracts of *E. coli*. Therefore, several sulfur compounds were investigated to determine whether they could serve as substrates for enzymatic biotin formation from DTB. As shown in Table I, only AdoMet was effective among the sulfur compounds tested. Although methionine has been reported to have a significant effect [106], it had no activity in my experiments.

Table I. Effects of Sulfur Compounds

The reaction mixture contained 40 mM Tris-HCl buffer (pH 8.0), 0.2 mM DL-DTB, the cell-free extracts (21 mg protein/ml) and the indicated sulfur compound at 1 mM.

Sulfur compounds	Biotin formed (nmol/mg•h)
None	0.01
L-Methionine	0.02
D-Methionine	0.01
L-Cysteine	0.02
D-Cysteine	0.01
DL-Homocysteine	0.01
S-Adenosyl-L-methionine	0.39
S-Adenosyl-L-homocysteine	0.01
Methylthioadenosine	0.02
S-Sulfocysteine	0.01
L-Cysteine sulfinic acid	0.01
L-Cysteinic acid	0.02
Cysteamine	0.02
Taurine	0.01
Glutathione (reduced)	0.01

The enzymatic reaction was done with the cell-free extracts containing various amounts of protein in the presence of 1 mM AdoMet. As shown in Fig. 1, the activity and the protein concentration bear a linear relationship to each other. However, in *B. sphaericus* cell-free extract, the enzymatic activity markedly increased as the protein concentrations were increased between 15 and 20 mg/ml as shown in Section 1 of this Chapter. Since it was thought that biotin synthase exists as a multienzyme complex, such a distinct correlation may suggest that *E. coli* and *B. sphaericus* have slightly different organization of biotin synthase.

The dialyzed cell-free extracts were prepared to exclude low molecular weight compounds. Then, no biotin synthase activity was detected in the dialyzed cell-free extracts as in those of *B. sphaericus*. Therefore, the enzymatic reaction was done by addition of various well-known cofactors.

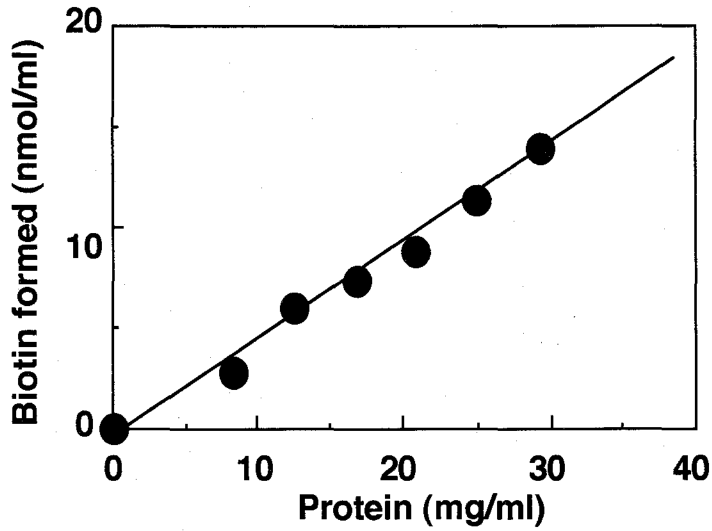


Fig. 1. Effect of Protein Concentration.
The reaction mixture contained 40 mM Tris-HCl buffer (pH 8.0), 0.2 mM DL-DTB, 1 mM AdoMet and the cell-free extract of the indicated protein concentrations.

Table II. Effects of Cofactors
The reaction mixture contained 40 mM Tris-HCl buffer (pH 8.0), 0.2 mM DL-DTB, 1 mM AdoMet and the dialyzed cell-free extracts (12 mg protein/ml) and the indicated compound at 1 mM.

Compounds	Biotin formed (nmol/mg•h)
None	0.003
NAD^+	0.013
NADH	0.014
$NADP^+$	0.078
NADPH	0.077
FAD	0.044
FMN	0.003
Pyrroloquinoline quinone	0.003
Cytochrome *c*	0.003
Phenazine ethosulfate	0.003
Pyridoxal 5'-phosphate	0.003
CoA (reduced form)	0.003

As shown in Table II, NAD^+, NADH, $NADP^+$, NADPH and FAD restored the activity. Especially, phosphate forms of pyridine nucleotide coenzymes, $NADP^+$ and NADPH, were significantly effective.

As mentioned above, only AdoMet was active among various sulfur compounds tested. Furthermore the effects of the same sulfur compounds indicated in Table I were examined in the presence of AdoMet again. Then, D- and L-cysteine evidently enhanced the enzyme activity (Table III). In Section 2 of this Chapter, it has been revealed that radioactive biotin was formed when cold AdoMet and [^{35}S] cysteine were added to the cell-free system of *B. sphaericus* while no radioactive biotin was produced from [^{35}S] AdoMet. In the present experiment, both D-and L-cysteine were active. It is not clear which isomer of cysteine is active , or why the reaction proceeded without addition of cysteine.

Table III. Effects of Sulfur Compounds in the Presence of AdoMet
The reaction mixture contained 40 mM Tris-HCl buffer (pH 8.0), 0.2 mM DL-DTB, 1 mM AdoMet, 1 mM NADPH, 1 mM FAD, and the cell-free extracts (23 mg protein/ml) and the indicated sulfur compound at 1 mM.

Sulfur compounds	Biotin formed (nmol/mg•h)
None	0.072
L-Methionine	0.072
D-Methionine	0.070
L-Cysteine	0.263
D-Cysteine	0.172
DL-Homocysteine	0.061
S-Adenosyl-L-homocysteine	0.038
Methylthioadenosine	0.069
S-Sulfocysteine	0.056
L-Cysteine sulfinic acid	0.052
L-Cysteinic acid	0.044
Cysteamine	0.072
Taurine	0.070
Glutathione (reduced)	0.086

SUMMARY

The activity of biotin synthesis from dethiobiotin was evidenced in cell-free extracts of an *Escherichia coli bioB* transformant. Among the sulfur compounds tested, only *S*-adenosyl-L-methionine had a significant effect, while methionine and cysteine were inert. The activity was linearly stimulated by increasing protein concentration. When the dialyzed cell-free extracts were used for the reaction, $NADP^+$, NADPH and FAD among the well-known cofactors tested promoted the activity. Furthermore, in the presence of AdoMet, cysteine was apparently effective toward biotin synthetic activity.

CONCLUSION

In this thesis, I investigated the microbial sulfur removal from dibenzothiophene and microbial sulfur insertion into biotin. The findings reported in each chapter are summarized as follows:

CHAPTER I

Rhodococcus erythropolis D-1 was isolated as a dibenzothiophene-degrading bacterium from soil. This strain could utilize dibenzothiophene as a sole source of sulfur. Dibenzothiophene was metabolized to 2-hydroxybiphenyl by the strain and 2-hydroxybiphenyl was almost stoichiometrically accumulated as the dead-end metabolite of dibenzothiophene degradation. Dibenzothiophene as an initial concentration of 0.125 mM was completely degraded within 2 days of cultivation. Dibenzothiophene up to 2.2 mM was rapidly degraded by resting cells within 150 min (Section 1).

The bacterium, *R. erythropolis* H-2, which can utilize dibenzothiophene as a sole source of sulfur in the presence of hydrocarbon, was isolated. When this strain was cultivated in a medium containing dibenzothiophene and *n*-tetradecane, dibenzothiophene was metabolized stoichiometrically to 2-hydroxybiphenyl within 1 day. This strain grew in the presence of *n*-octane and longer carbon-chain hydrocarbons. Dibenzothiophene degradation proceeded in the resting cells system with lyophilized cells of this strain. The addition of *n*-tetradecane enhanced the reaction rate, the optimal concentration of *n*-tetradecane was 40% (Section 2).

R. erythropolis H-2 can also grow on dibenzothiophene derivatives, 2,8-dimethyl dibenzothiophene and 4,6-dimethyl dibenzothiophene. In the resting cells reaction, these dibenzothiophene derivatives added at 1 mM were completely degraded within 6 h as was dibenzothiophene. The reaction products were assumed to be the monohydroxy biphenyl form of corresponding dibenzothiophene derivatives (Section 3).

These two bacteria are believed to be significantly important for the microbial desulfurization of petroleum because both of them only act on sulfur atom of dibenzothiophene and one strain, *R. erythropolis* H-2, works even in the presence of hydrocarbon and acts on more complexed dibenzothiophene as well as dibenzothiophene.

Chapter II

The enzymatic desulfurization of dibenzothiophene to 2-hydroxybiphenyl was detected for the first time in cell-free extracts of *R. erythropolis* D-1 grown on dibenzothiophene as a sole source of sulfur. In the dialyzed cell-free extracts, NADH was absolutely required for the activity. Dibenzothiophene desulfurization proceeded linearly with the reaction time and stoichiometric amounts of 2-hydroxybiphenyl were finally formed (Section 1).

In the process of the purification of a dibenzothiophene-degrading enzyme system from cell-free extracts of *R. erythropolis* D-1, flavin coenzymes, FMN and FAD, were found to be involved in the enzymatic degradation of dibenzothiophene. Under these experimental conditions, the optimal concentrations of FMN and FAD were both 10 μM and the activity was completely inhibited by the addition of 1 mM FMN or FAD. Dibenzothiophene was converted to dibenzothiophene sulfone stoichiometrically and 2-hydroxybiphenyl formation was not observed when the reaction was done using the enzyme preparation purified by DEAE-Sepharose column chromatography (Section 2).

The enzymatic activity involved in dibenzothiophene degradation of *R. erythropolis* D-1 was found in cell-free extracts of cells grown not only with dibenzothiophene as a sole sulfur source but also with its analogs, thioxanthen-9-one and dibenzothiophene sulfone. The enzyme formation was completely repressed in a medium with 0.5 mM sodium sulfate or 0.1 mM methanesulfonic acid even in the presence of dibenzothiophene. The enzyme activity in the cell-free extracts of this strain was inhibited by a degradation product, 2-hydroxybiphenyl, and its analog, 2,2'-dihydroxybiphenyl, but not by sodium sulfate and biphenyl (Section 3).

The enzyme proteins in the dibenzothiophene oxygenase system of *R. erythropolis* D-1 involved in the conversion of dibenzothiophene to dibenzothiophene sulfone were separated into two fractions (fraction A and fraction B) at the purification stage by DEAE-Sepharose column chromatography. One enzyme protein, designated as component B, was purified to homogeneity. The molecular mass of the component B was 250 kDa and it consisted of identical six subunits with a molecular weight of 45 kDa. Another enzyme protein(s) contained in fraction A has NADH-linked reductase activity with artificial electron acceptors. The dibenzothiophene oxygenase system exhibited a narrow substrate specificity, it acted on some dibenzothiophene derivatives but not on carbazole and fluorene. A analyses using some inhibitors suggested that thiol group and metal are related to the enzyme activity (Section 4).

These findings are significant for the elucidation of microbial or enzymatic sulfur removal from the aromatic sulfur compound.

Chapter III

The activity of biotin synthase, responsible for biotin synthesis from dethiobiotin, was demonstrated in a completely defined reaction mixture with cell-free extracts of a *Bacillus sphaericus bioB* transformant. Among the sulfur compounds tested, only *S*-adenosyl-L-methionine was active, while L-methionine and L-cysteine had no significant effect. When dialyzed cell-free extracts were used for the reaction, NADH, NADPH, and FAD among the well-known cofactors tested enhanced the activity (Section 1)

In a cell-free system of a *bioB* transformant of *B. sphaericus*, the introduction of sulfur into dethiobiotin was investigated using sulfur labelled compounds. By using [^{35}S] *S*-adenosylmethionine and [^{35}S] cysteine, the sulfur donor was suggested to be cysteine or its derivatives not *S*-adenosylmethionine. This finding together with the fact that NADPH and FAD are required for activity suggests some analogy between the biotin synthase and other systems which use *S*-adenosylmethionine as a source of deoxyadenosyl radical (Section 2).

The activity of biotin synthesis from dethiobiotin was also evidenced in cell-free extracts of an *Escherichia coli bioB* transformant. Requirements for *S*-adenosyl-L-methionine and other cofactors were similar to those of *B. sphaericus*. In the presence of AdoMet, cysteine was apparently effective toward biotin synthetic activity (Section 3).

These findings may shed light on the sulfur insertion reactions for other living materials.

ACKNOWLEDGMENTS

I wish to express my sincere thanks to Professor Sakayu Shimizu, Kyoto University, for his kind guidance and encouragement throughout the course of this work.

I am deeply indebted to Professor Yoshikazu Izumi, Tottori University, without whose thoughtful advice and instruction this work would not have been possible.

I am also grateful to Emeritus Professor Hideaki Yamada, Kyoto University, (Professor of Toyama Prefectural University) for his kind suggestion and encouragement.

Special thanks are due to Professor Andrée Marquet, Dr. Dominique Florentin and Dr. Bernadette T. S. Bui, Université Paris-VI, for their great collaborations.

I would like to express my thanks to the members of Shimizu laboratory of the Petroleum Energy Center subsidized from the Ministry of International Trade and Industry for their valuable advice.

I greatly appreciate all members of our laboratory in Tottori University for their help in this study.

REFERENCES

1) Kinsel, N. A. (1960) *J. Bacteriol.*, **80,** 628-632

2) Sugio, T., Domatsu, C., Tano, T., and Imai, K. (1984) *Appl. Environ. Microbiol.*, **48,** 461-467

3) Unz, R. F., and Lundgren, D. G. (1961) *Soil. Sci.*, **92,** 302-313

4) Charles, A. M., and Suzuki, I. (1966) *Biochim. Biophys. Acta,* **128,** 522-534

5) Silver, M., and Lundgren, D. G. (1968) *Can. J. Biochem.*, **46,** 457-461

6) Suzuki, I. (1965) *Biochim. Biophys. Acta,* **104,** 359-371

7) Suzuki, I., and Silver, M. (1966) *Biochim. Biophys. Acta,* **122,** 22-23

8) Sugio, T., Mizunashi, W., Inagaki, K., and Tano, T. (1987) *Appl. Environ. Microbiol.*, **169,** 4916-4922

9) Garcia, J. J., and Maitlis, P. M. (1993) *J. Am. Chem. Soc.*, **115,** 12200-12201

10) Garcia, J. J., Mann, B. E., Adams, H., Bailey, N. A., and Maitlis, P. M. (1995) *J. Am. Chem. Soc.*, **117,** 2179-2186

11) Koczaja Dailey, K. M., Rauchfuss, T. B., Rheingold, A. L., and Yap, G. P. A. (1995) *J. Am. Chem. Soc.*, **117,** 6396-6397

12) Detz, C. M., and Barvinchak, G. (1979) *Mining Congr. J.*, **65,** 75-82

13) Kargi, F., and Robinson, J. M. (1985) *Biotechnol. Bioeng.*, **27,** 41-49

14) Elogwe, C. A. (1988) *FUEL*, **67,** 451-458

15) Ohmura, N., Kitamura, K., and Saiki, H. (1993) *Biotechnol. Bioeng.*, **41,** 671-676

16) Ohmura, N., Kitamura, K., and Saiki, H. (1993) *J. Bacteriol.*, **59,** 4044-4050

17) Kargi, F., and Robinson, J. M. (1986) *FUEL*, **65,** 397-399

18) Runnion, K., and Combie, J. D. (1993) *FEMS Microbiol. Rev.*, **11,** 139-144

19) Karavaiko, G. I., and Lobyreva, L. B. (1994) *Fuel Process Technol.*, **40,** 167-182

20) Knowles, J. R. (1989) *Annu. Rev. Biochem.*, **58,** 195-221

21) Dakshinamurti, K., Bhaganan, H., Eds. (1985) *Ann. N. Y. Acad. Sci.*, **447,** 1-441

22) Gyoerg, P., and Lenger, B. W. (1968) *The vitamins.* Academic Press, New York, pp 279-280

23) Deroose, F. D., and De Clercq, P. J. (1995) *J. Org. Chem.*, **60,** 321-330

24) Deroose, F. D., and De Clercq, P. J. (1994) *Tetrahedron Lett.*, **35,** 2615-2618

25) Yamada, H., Osakai, M., Tani, Y., and Izumi, Y. (1983) *Agric. Biol. Chem.*, **47,** 1011-1016

26) Sabatié, J., Speck, D., Reymund, J., Hebert, C., Caussin, L., Weltin, D., Gloeckler, R., O'Regan, M., Bernard, S., Ledoux, C., Ohsawa, I., Kamogawa, K., Lemoine, Y., and Brown, S. W. (1991) *J. Biotechnol.*, **20,** 29-50

27) Masuda, M., Takahashi, K., Sakurai, N., Yanagiya, K., Komatsubara, S., and Tosa, T. (1995) *Process Biochem.*, **30,** 553-562

28) Izumi, Y., Morita, H., Tani, Y., and Ogata, K. (1974) *Agric. Biol. Chem.*, **38,** 2257-2262

29) Ifuku, O., Miyaoka, H., Koga, N., Kishimoto, J., Haze, S., Wachi, Y., and Kajiwara, M. (1994) *Eur. J. Biochem.*, **220,** 585-591

30) Sanyal, I., Lee, S-L., and Flint, D. H. (1994) *J. Am. Chem. Soc.*, **116,** 2637-2638

31) Izumi, Y., Kano, Y., Inagaki, K., Kawase, N., Tani, Y., and Yamada, H. (1981) *Agric. Biol. Chem.*, **45,** 1983-1989

32) Ploux, O., Soularue, P., and Marquet, A. (1992) *Biochem. J.*, **287,** 685-690

33) Ploux, O., and Marquet, A. (1992) *Biochem. J.*, **283,** 327-331

34) Stoner, G. L., and Eisenberg, M. A. (1975) *J. Biol. Chem.*, **250,** 4036-4039

35) Yang, H-C., Tani, Y., and Ogata, K. (1971) *Agric. Biol. Chem.*, **35,** 1346-1352

36) van Afferden, M., Schacht, S., Klein, J., and Truper, H. G. (1990) *Arch. Microbiol.*, **153,** 324-328

37) Monticello, D. J., Bakker, D., and Finnerty, W. R. (1985) *Appl. Environ. Microbiol.*, **49,** 756-760

38) Laborde, A. L., and Gibson, D. T. (1977) *Appl. Environ. Microbiol.*, **34,** 783-790

39) Yeong, K. H., Kim, T. S., and Kim, B. H. (1990) *Biotechnol. Lett.*, **12,** 761-764

40) Mormile, M. R., and Atlas, R. M. (1988) *Appl. Environ. Microbiol.*, **54,** 3183-3184

41) Omori, T., Monna, L., Saiki, Y., and Kodama, T. (1992) *Appl. Environ. Microbiol.*, **58,** 911-915

42) Gallagher, J. R., Olson, E. S., and Stanley, D. C. (1993) *FEMS Microbiol. Lett.*, **107,** 31-36

43) Dodgson, K. S. (1961) *Biochem. J.*, **78,** 312-319

44) Kodama, K., Nakatani, S., Umehara, K., Shimizu, K., Minoda, Y., and Yamada, K. (1970) *Agric. Biol. Chem.*, **34,** 1320-1324

45) Olson, E. S., Stanley, D. C., and Gallagher, J. R. (1993) *Energy Fuels*, **7,** 159-164

46) Kayser, K. J., Bielaga-Jones, B. A., Jackowski, K., Odusan, O., and Kilbane II, J. J. (1993) *J. Gen. Microbiol.*, **139,** 3123-3129

47) Wang, P., and Krawiec, S. (1994) *Arch. Microbiol.*, **161,** 266-271

48) Denome, S. A., Olson, E. S., and Young, K. D. (1993) *Appl. Environ. Microbiol.*, **59,** 2837-2843

49) Hansh, C., Muir, M. R., Fujita, T., Maloney, P. P., Geiger, F., and Streich, M. (1963) *J. Am. Chem. Soc.*, **85,** 2817-2824

50) Hansh, C., and Fujita, T. (1964) *J. Am. Chem. Soc.*, **86,** 1616-1626

51) Inoue, A., and Horikoshi, K. (1991) *J. Ferment. Bioeng.*, **71,** 194-196

52) Kilbane II, J. J., and Bielaga, B. A. (1990) *Chemtech*, **20,** 747-751

53) Kilbane II, J. J., and Jackowski, K. (1992) *Biotechnol. Bioeng.*, **40,** 1107-1114

54) Constantí, M., Giralt, J., and Bordons, A. (1994) *World Journal of Microbiology & Biotechnology*, **10,** 510-516

55) Gerdil, R., and Lucken, E. A. C. (1965) *J. Am. Chem. Soc.*, **87,** 213-217

56) Bradford, M. (1976) *Anal. Biochem.*, **72,** 248-254

57) Locher, H. H., Leisinger, T., and Cook, A. M. (1991) *Biochem. J.*, **274,** 833-842

58) Junker, F., Leisinger, T., and Cook, A. M. (1994) *Microbiology U. K.*, **140,** 1713-1722

59) Denome, S. A., Oldfield, C., Nash, L. J., and Young, K. D. (1994) *J. Bacteriol.*, **176,** 6707-6716

60) Haiger, B. E., and Gibson, D. T. (1990) *J. Bacteriol.*, **172,** 457-464

61) Gunsalus, I. C., and Wagner, G. C. (1978) *Methods in Enzymol.*, **52,** 166-168

62) Subramanian, V., Liu, T.-N., Yeh, W.-K., Narro, M., and Gibson, D. T. (1981) *J. Biol. Chem.*, **256,** 2723-2730

63) van Berkel, W. J. H., and Müller, F. (1991) Chemistry and Biochemistry of Flavoenzymes, Vol. II, ed. by F. Müller, CRC Press, London, pp 1-30

64) Omori, T., Saiki, Y., Kasuga, K., and Kodama, T. (1995) *Biosci. Biotech. Biochem.*, **59**, 1195-1198

65) Kertesz, M. A., Cook, A. M., and Leisinger, T. (1994) *FEMS Microbiol. Rev.*, **15**, 195-215

66) Kertesz, M. A., Leisinger, T., and Cook, A. M. (1993) *J. Bacteriol.*, **175**, 1187-1190

67) Harada, T. (1964) *Biochim. Biophys. Acta,* **81**, 193-196

68) Harada, T., and Spencer, B. (1964) *Biochem. J.*, **93**, 373-378

69) Adachi, T., Okamura, H., Murooka, Y., and Harada, T. (1974) *J. Bacteriol.*, **120**, 880-885

70) Adachi, T., Murooka, Y., and Harada, T. (1975) *J. Bacteriol.*, **121**, 29-35

71) Azakami, H., Sugio, H., and Murooka, Y. (1992) *J. Bacteriol.*, **174**, 2344-2351

72) Ikeda, Y., and Tanaka, K. (1983) *J. Biol. Chem.*, **258**, 1077-1085

73) Kim, J-J. P., Wang, M., and Paschke, R. (1993) *Proc. Natl. Acad. Sci. USA,* **90**, 7523-7527

74) Armstrong, J. M. (1964) *Biochim. Biophys. Acta,* **86**, 194-197

75) Massey, V. (1959) *Biochim. Biophys. Acta,* **34**, 255-256

76) Schellenberg, K. A., and Hellerman, L. (1958) *J. Biol. Chem.*, **231**, 547-556

77) Laemmli, U. K. (1970) *Nature*, **227**, 680-685

78) Markus, A., Krekel, D., and Lingens F. (1986) *J. Biol. Chem.*, **261**, 12883-12888

79) Batie, C. J., LaHaie, E., and Ballou, D. P. (1987) *J. Biol. Chem.*, **262,** 1510-1518

80) Schweizer, D., Markus, M., Seez, M., Ruf, H. H., and Lingens F. (1987) *J. Biol. Chem.*, **262,** 9340-9346

81) Fetzner, S., Müller, R., and Lingens, F. (1992) *J. Bacteriol.*, **174,** 279-290

82) Piddington, C. S., Kovacevich, B. R., and Rambosek, J., (1995) *Appl. Environ. Microbiol.*, **61,** 468-475

83) Izumi, Y., and Ogata, K. (1977) *Adv. Appl. Microbiol.*, **22,** 145-176

84) Izumi, Y., Tani, Y., and Ogata, K. (1979) *Methods in Enzymol.*, **62,** 326-338

85) Eisenberg, M. A., and Stoner, G. L. (1979) *Methods in Enzymol.*, **62,** 342-347

86) Eisenberg, M. A., and Krell, K. (1979) *Methods in Enzymol.*, **62,** 348-352

87) Izumi, Y., Tani, Y., and Yamada, H. (1980) *Bull. Inst. Chem., Kyoto Univ.*, **58,** 434-447

88) Parry, R. J., and Kunitani, M. G. (1976) *J. Am. Chem. Soc.*, **98,** 4024-4026

89) Guillerm, G., Frappier, F., Gaudry, M., and Marquet, A. (1977) *Biochimie,* **59,** 119-121

90) Salib, A. G., Frappier, F., Guiierm, G., and Marquet, A. (1979) *Biochem. Biophys. Res. Commun.*, **88,** 312-319

91) Parry, R. J., and Naidu, M. V. (1980) *Tetrahedron Lett.*, **21,** 4783-4786

92) Demoll, E., and Shive, W. (1983) *Biochem. Biophys. Res. Commun.*, **110,** 243-249

93) Demoll, E., White, R. H., and Shive, W. (1984) *Biochemistry,* **23,** 558-562

94) Even, L., Florentin, D., and Marquet, A. (1990) *Bull. Soc. Chem. Fr.*, **127,** 758-768

95) Baxter, R. L., Camp, D. J., Coutts, A., and Shaw, N. J. (1992) *J. Chem. Soc. Perkin Trans.*, **1,** 255-258

96) Marquet, A., Frappier, F., Guillerm, G., Azoulay, M., Florentin, D., and Tabet, J-C. (1993) *J. Am. Chem. Soc.*, **115,** 2139-2145

97) Baldet, P., Gerling, H., Axiotis, S., and Douce, R. (1993) *Eur. J. Biochem.*, **217,** 479-485

98) Cleary, P. P., and Campbell, A. (1972) *J. Bacteriol.*, **112,** 830-839

99) Otsuka, A. J., Buoncristiani, M. R., Howard, P. K., Flamm , J., Johnson, C., Yamamoto, R., Uchida, K., Cook, C., Ruppert, J., and Matsuzaki, J. (1988) *J. Biol. Chem.*, **263,** 19577-19585

100) Gloeckler, R., Ohsawa, I., Speck, D., Ledoux, C., Bernard, S., Zinsus, M., Villeval, D., Kisou, T., Kamogawa, K., and Lemoine, Y. (1990) *Gene* , **87,** 63-70

101) Brown, S. W., Speck, D., Sabatie, J., Gloeckler, R., O'Regan, M., Viret, J. F., and Lemoine, L. (1991) *J. Chem. Tech. Biotechnol.*, **50,** 115-121

102) Ohsawa, I., Kisou, T., Kodama, K., Yoneda, I., Speck, D., Gloeckler, R., Lemoine, Y., and Kamogawa, K. (1992) *J. Ferment. Bioeng.*, **73,** 121-124

103) Levy-Schil, S., Debussche, L., Rigault, S., Soubrier, F., Bacchetta, F., Lagneaux, D., Schleuniger, J., Blanche, F., Crouzet, J., and Mayaux, J-F. (1993) *Appl. Microbiol. Biotechnol.*, **38,** 755-762

104) Sakurai, N., Imai, Y., Masuda, M., Komatsubara, S., and Tosa, T. (1993) *Appl. Environ. Microbiol.*, **59,** 3225-3232

105) Ohsawa, I., Speck, D., Kisou, T., Hayakawa, K., Zinsus, M., Gloeckler, R., Lemoine, Y., and Kamogawa, K. (1989) *Gene*, **80,** 39-48

106) Ifuku, O., Kishimoto, J., Haze, S., Yanagi, M., and Fukushima, S. (1992) *Biosci. Biotech. Biochem.*, **56,** 1780-1785

107) Fujisawa, A., Abe, T., Ohsawa, I., Shiozaki, S., Kamogawa, K., and Izumi, Y. (1993) *Biosci. Biotech. Biochem.*, **57,** 740-744

108) Fujisawa, A., Abe, T., Ohsawa, I., Kamogawa, K., and Izumi, Y. (1993) *FEMS Microbiol. Lett.*, **110,** 1-4

109) Tanaka, M., Izumi, Y., and Yamada, H. (1987) *J. Microbiol. Methods*, **6,** 237-246

110) Niimura, T., Suzuki, T., and Sahashi, Y. (1964) *J. Vitaminol.*, **10,** 231-236

111) Izumi, Y., Sugisaki, K., Tani, Y., and Ogata, K. (1973) *Biochim., Biophys. Acta,* **304,** 887-890

112) Markham, G. D., Hafner, E. W., Tabor, C. W., Tabor, H. (1980) *J. Biol. Chem.*, **255,** 9082-9092

113) Eliasson, R., Pontis, E., Fontecave, M., Gerez, C., Harder, J., Jornvall, H., Krook, M., and Reichard, P. (1992) *J. Biol. Chem.*, **267,** 25541-25547

114) Harder, J., Eliasson, R., Pontis, E., Ballinger, M. D., and Reichard, P. (1992) *J. Biol. Chem.*, **267,** 25548-25552

115) Mulliez, E., Fontecave, M., Gaillard, J., and Reichard, P. (1993) *J. Biol. Chem.*, **268,** 2296-2299

116) Wagner, A. F. V., Frey, M., Neuberger, F. A., Schafer, W., and Knappe, J. (1992) *Proc. Natl. Acad. Sci. U.S.A.*, **89,** 996-1000

117) Wong, K. K., Murray, B. W., Lewisch, S. A., Baxter, M. K., Ridky, T. W., Ullssi-Demario, L., and Kozarich, J. W. (1993) *Biochemistry*, **32**, 14102-14110

118) Petrovich, M. R., Ruzicka, F. J., Reed, G. H., and Frey, P. A. (1992) *Biochemistry*, **31**, 10774-10781

119) Sanyal, I., and Flint, D. (1993) *J. Inorg. Biochem.*, **51**, 505

120) Sanyal, I., Cohen, G., and Flint, D. H. (1994) *Biochemistry*, **33**, 3625-3631

121) Ifuku, O., Koga, N., Haze, S., Kishimoto, J., and Wachi, Y. (1994) *Eur. J. Biochem.*, **224**, 173-178

PUBLICATIONS

CHAPTER I

Section 1

Izumi, Y., Ohshiro, T., Ogino, H., Hine, Y., and Shimao, M. (1994) *Appl. Environ. Microbiol.*, **60**, 223-226

Section 2

Ohshiro, T., Hirata, T., and Izumi, Y. (1995) *Appl. Microbiol. Biotechnol.*, **44**, 249-252

Section 3

Ohshiro, T., Hirata, T., and Izumi, Y. submitted for publication

CHAPTER II

Section 1

Ohshiro, T., Hine, Y., and Izumi, Y. (1994) *FEMS Microbiol. Lett.*, **118**, 341-344

Section 2

Ohshiro, T., Kanbayashi, Y., Hine, Y., Izumi, Y. (1995) *Biosci. Biotech. Biochem.*, **59**, 1349-1351

Section 3

Ohshiro, T., Suzuki, K., and Izumi, Y. (1996) *J. Ferment. Bioeng.*, **81**, 121-124

Section 4

Ohshiro, T., Suzuki, K., and Izumi, Y. submitted for publication

CHAPTER III

Section 1

Ohshiro, T., Yamamoto, M., Izumi, Y., Bui, B. T. S., Florentin, D., and Marquet, A. (1994) *Biosci. Biotech. Biochem.*, **58**, 1738-1741

Section 2

Florentin, D., Bui, B. T. S., Marquet, A., Ohshiro, T., and Izumi, Y. (1994) *C. R. Acad. Sci. Paris, Science de la vie,* **317**, 485-488

Section 3

Ohshiro, T., Yamamoto, M., Bui, B. T. S., Florentin, D., Marquet, A., and Izumi, Y. (1995) *Biosci. Biotech. Biochem.*, **59**, 943-944